ADVANCES IN HORTICULTURAL SCIENCE

Formerly Rivista dell'Ortoflorofrutticoltura Italiana
founded in 1876 and issued by University of Florence, Italy

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Advances in Horticultural Science is published by the Department of Agri-Food Production and Environmental Sciences, University of Florence, Viale delle Idee, 30, 50019 Sesto Fiorentino (FI), Italy
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Adv. Hort. Sci., 2021 35(3): 217-231 DOI: 10.36253/ahsc-10706

AHS
Advances in Horticultural Science

Punica granatum L. extract contributes to phytopathogens control and enhances *Eruca vesicaria* (L.) Cav. germination *in vitro* and *in vivo*

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Citation:
DE SOUSA SILVA S., ALVES P.C.S., COUTINHO D.F., LUZ T.R.S.A., FONTOURA G.M.G., BERRETTA A.A., GONÇALVES MACIEL M.C., 2021 - *Punica granatum L. extract contributes to phytopathogens control and enhances Eruca vesicaria (L.) Cav. germination in vitro and in vivo.* - Adv. Hort. Sci., 35(3): 217-231.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 20 March 2021
Accepted for publication 26 May 2021

Key words: antimicrobial, botanical pesticides, food security, plant diseases, pomegranate peel, punicalagin.

Abstract: The study aimed to investigate antimicrobial activity of the hydroalcoholic crude extract from the fruit peel of *Punica granatum* (Pp) and punicalagin compound (Pg) on phytopathogenic bacterial isolates and its potential use as a sustainable alternative in treatment of vegetable seeds. The antimicrobial activity *in vitro* was tested by agar well diffusion assay and through viability tests in liquid medium. *In vivo* treatment with Pp was tested on *Eruca vesicaria* seeds infected with *Xanthomonas campestris* pv. *campestris.* Pp induced the formation of large inhibition zones to the growth of the tested pathogens (35.33 mm - 6.66 mm), with dose-dependent effect. Viability tests confirmed the antimicrobial activity of the Pp on *X. campestris* pv. *campestris* and *P. carotovorum* subsp. *carotovorum* with minimum inhibitory concentration (MIC) of 125 µg/mL. Punicalagin compound presented MIC of the 31.25 µg/mL. The seed treatment with Pp indicated control of pathogen-induced symptoms in seedlings of the *E. vesicaria* and positive effect in seed germination, emergence and in stomatal functionality. The results indicate strong potential of the extract from the fruit peel of *P. granatum* and Punicalagin for formulating botanical pesticides for plant disease control.

1. Introduction

According to Food and Agriculture Organization of the United Nations estimates, by 2050 the world population should exceed 9.5 billion inhabitants, raising the demand for food by up to 60% (FAO, 2016). Plants account for 80% of food ingested in the human diet, providing affordable, safe and nutritious resources for a healthy life. However, pests and diseases pose a threat to food security, due to damage caused to crops that compromises access to food and rises product prices (FAO, 2017).

Phytopathogenic bacteria causes a large number of different plant diseases, some of which are devastating to agricultural crops (Van Der Wolf and De Boer, 2015). *Ralstonia solanacearum* (Smith) (Yabuuchi *et al*., 1995) stands out as one of most destructive pathogens due to the rapid development of wilting symptoms and death of host plants (Yuliar *et al.,* 2015). The pathogen affects a large range of host plants, comprising almost 450 species from 54 different botanical families (Allen *et al.,* 2005).

Direct losses in important crops are estimated in 0 to 91% (tomatoes) and in 33 to 90% in potatoes (Elphinstone, 2005). The bacterium *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson is a vascular (Ryan *et al.,* 2011) and seed-born (Griesbach *et al.,* 2003) pathogen which is distributed worldwide. Infested seeds may emerge in young seedlings infected by the pores on the margin of the cotyledons. This pathogen causes black rot disease, which seriously affects Brassicaceae (Cruciferous) crops (Vicente and Holub, 2013), important food items grown worldwide (Gupta *et al.,* 2013). Finally, *Pectobacterium* is widely studied soft-rot bacterial pathogen causing infections in potato crops and stored tubers, reducing the production and quality of tubers (Adeolu *et al.,* 2016). *Ralstonia solanacearum*, *X. campestris* and *P. carotovorum* have been included among the 10 most important bacterial pathogens of the plants according to their economic and scientific impact (Mansfield *et al.,* 2012).

Control of bacterial diseases in conventional agriculture often uses fast-acting synthetic pesticides and antimicrobials (Kotan *et al.,* 2014). According to national phytosanitary pesticide database (AGROFIT, 2016), substances unsafe to the environment like kasugamycin, cuprous oxide, copper hydroxide and 'extremely toxics' like benzalkonium chloride were registered for control of soft rot *P. carotovorum* subsp. *carotovorum* in potatoes. Pesticides indicated for control of bacterial wilt caused by *R. solanacearum* like Bismerthiazol and Thiodiazole copper have shown low efficacy, high phytotoxicity, harmful environmental effects and bacterial resistance development (Yang and Bao, 2017).

Cultural practices like the use of pathogen-free seeds is recommended to prevent black rot disease in crops (Chitarra *et al.,* 2002). If pathogen-free seed is not available, seed should be treated to eliminate the bacteria. However, seed treatments do not always eliminate 100% of bacteria on or in the seed, and may adversely affect seed germination and vigor (Celetti and Callow, 2002).

The need to reduce chemical pesticide use in crops, associated with demands for healthy food and development of sustainable agriculture, has driven research for natural compounds with low impact on the environment and on people health (Jiménez-Reyes *et al.,* 2019). The secondary metabolism of the plants produces many bioactive compounds that provide protection against pests and pathogens (Borges *et al.,* 2018). Unlike synthetic pesticides, natural compounds exhibit rapid biodegradation after use in the field (Soberón *et al.,* 2014), little or no phytotoxicity, abundant sources and low costs, since they come from a renewable source (Zheng *et al.,* 2016). Thus, medicinal plant uses with antimicrobial activity can be considered an effective component in the integrated management against phytopathogens (Khan *et al.,* 2020).

Punica granatum L. (Pomegranate) is a plant of the Lythraceae family, native from central Asia (northern India to Iran), nowadays cultivated in several parts of the world, including Africa and America (Viuda-Martos *et al.,* 2010; Erkan and Dogan, 2018). The fruit of Pomegranate (called balausta) is a pulp berry formed by a thick and leathery skin with variable color depending on the variety. The seeds are a reproductive structure that present a fleshy outer testa called sarcotesta where the juice is extracted (Melgarejo *et al*., 2020). The production, marketing and consumption of pomegranate fruit have increased rapidly throughout the world in recent years, mainly due to greater awareness of their health-promoting attributes (Selcuk and Erkan, 2015).

The peel of *P. granatum* represents 30 to 40% of the fruit, being usually discarded as waste during industrial processing for the production of pomegranate juice (Gullon *et al.,* 2016). However, this part of the fruit is rich in phenolic acids, tannins (such as punicalin and punicalagin) and flavonoids with vari-

ous biological functions, including activity against pathogenic microorganisms (Dey *et al.,* 2012; Türkyılmaz *et al.*, 2013). Punicalagin compound is an important bioactive agent found in pomegranate fruit peel, with antioxidant, antimicrobial, antiviral and immunosuppressive activity. The compound belongs to the ellagitannin family which includes other tannins such as punicalin and gallic acid, characterized by good water solubility (Akhtar *et al.,* 2015).

In the last years several studies have evidenced the antimicrobial activity of the pomegranate extract against many species of the plant pathogenic fungi suggesting high potential source of natural antifungal agents (Mohamad and Khalil, 2015; Balah and Nowra, 2016; Elsherbiny *et al.,* 2016; Li Destri Nicosia *et al.,* 2016; Rongai *et al.,* 2017; Karm, 2019; El Khetabi *et al.,* 2020). However, few studies have investigated the antimicrobial activity of the pomegranate extract against phytopathogenic bacteria (Quattrucci *et al.,* 2013; Farag *et al.,* 2015; Khaleel *et al.,* 2016). Khaleel *et al.* (2016) have indicated *in vitro* antimicrobial activity of the ethyl acetate pomegranate peel extract against *R. solanacearum*, *P. carotovorum* subsp. *carotovorum* and *X. gardineri* and Farag *et al.* (2015) highlighted notable *in vitro* antimicrobial activities from the methanol pomegranate peel extract against variety of temperate climate (race 3, biovar 2) of the *R. solanacearum*. Despite this, the knowledge about the antimicrobial activity of the hidroalcoholic extract of *P. granantum* and isolate compounds against these pathogens is scarce. Thus, this study aimed to investigate the *in vitro* antimicrobial activity of hydroalcoholic crude extract from the fruit peel of the *P. granatum* and Punicalagin compound on isolates of phytopathogenic bacteria. The potential of the extract for natural control of *X. campestris* pv. *campestris* as an sustainable alternative for treatment of vegetable seeds was assayed.

2. Materials and Methods

Bacterial Isolates

The bacterial isolates were provided by the collection of the São Paulo Biological Institute - São Paulo, Brazil (*X. campestris* pv. *campestris*, Isolate No. Xcc2149) and Rosa Mariano Culture Collection of the Federal Rural University of Pernambuco, Brazil (*R. solanacearum*, Isolate No. CCRMRs187, race 3, biovar 1; and *P. carotovorum* subsp. *carotovorum*, Isolate No. CCRMPcc36). Agar medium of 523 Kado & Hesket was used as a culture medium for maintenance of the microbial cultures before antimicrobial tests.

Hydroalcoholic crude extract from the fruit peel of the P. granatum and pure compound

The hydroalcoholic crude extract from the fruit peel of the *P. granatum* (Pp) was supplied by Apis Flora®. The hydroalcoholic crude extract was concentrated under low pressure, dried and the remainder was later lyophilized. For the experiments, the lyophilized dry residue was diluted in an isotonic phosphate buffered saline (PBS). The Pp was concentrated under reduced pressure. Pure Punicalagin (Pg) compound was purchased from Sigma-Aldrich Brazil Ltda (P0023, 1 mg, Batch: WXBC5016V), ≥98% (HPLC), of pomegranate, $C_{48}H_{28}O_{30}$, molecular weight of 1084.72.

Chemical characterization of the hydroalcoholic crude extract from the fruit peel of the P. granatum

Total phenolic compounds. For determination of total phenolics an analytical curve of tannic acid (Sigma-Aldrich) was carried out. Pomegranate extract was prepared in 50 mL volumetric flask using water as solvent. The samples were homogenized and, the flasks were brought to the ultrasonic bath for 30 minutes. A 0.5 mL aliquot was transferred to another 50 mL flask where 2.5 mL of Folin-Denis reagent and 5.0 mL of 29% sodium carbonate were added. The samples were protected from the light and the readings were performed after 30 minutes in a UV-Vis spectrophotometer at 760 nm (Fernandes *et al*., 2018). All samples were analyzed in triplicate.

Ellagic acid. Ellagic acid (EA) was acquired from Fluka (95.0%, Batch BCBN4398V). The High-Performance Liquid Chromatography (HPLC) grade methanol was supplied by J.T. Baker (Mexico City, Mexico), and purified water was obtained using a Milli-Q Direct Q-5 filter system (Millipore, Bedford, USA). The analytical grade acetic acid was purchased from Synth (Labsynth, Diadema, Brazil).

To determine the ellagic acid content (EAC), the extracts previously diluted in methanol were properly homogenate using a vortex and then remained for 30 minutes in ultrasound bath. The solution was filtered and subjected to HPLC analysis (Shimadzu apparatus equipped with a CBM controller, LC-20AT quaternary pump, a SPD-M 20A diode-array detector and auto sampler, Shimadzu LC solution software,

version 1.21 SP1) using a 100 mm x 2.6 mm Shim pack ODS C18 column.

The mobile phase used for ellagic acid was methanol and acetic acid aqueous solution 2% using a elution gradient (0-7 min, 20-72.5% v/v methanol, 7-7.5 min, 72.5-95% v/v methanol, 7.5-8.5 min. 95% v/v methanol, 8.5-9 min 95-20% v/v methanol, 9-10 min 20% v/v methanol) with a flow rate of 1.0 mL min^{-1}, and oven temperature of 25°C. The eluted samples were detected by UV detector at 254 nm. Calibration curve was constructed by plotting the peak area (y) against concentration in µg mL^{-1} of standard solutions (x). The standard equation obtained from the curve was used for quantification of ellagic acid as mg/g extract of sample. All assays were carried out in triplicates and the ellagic acid quantification was reported.

Determination of antimicrobial activity

The Pp was assayed for antibacterial activity at different concentrations using a standard agar-well diffusion assay (CLSI, 2012). Suspensions of bacteria strains (1.5 x 10^8 CFU/mL) were spread using swabs over the 523 Kado & Hesket agar media in sterilized Petri dishes. Then, wells with a diameter of 6 mm were punched aseptically and 25 µL of Pp at different concentrations were introduced into each well (100, 50, 25, 12.5, 6.25 and 3.125 mg/mL, solubilized in an isotonic phosphate buffered saline (PBS). All plates were incubated at 28°C for 48 hours. Measures of the zones around the wells (mm) were recorded as inhibition zone for Pp. Streptomycin sulfate (500 µg/ml, *P. carotovorum* subsp. *carotovorum*) (Pachupate and Kininge, 2013) and oxytetracyclin hydrochloride (Terramicin®) (30 µg/mL, *X. campestris pv. campestris* and *R. solanacearum*) (Santos *et al.,* 2008) were used as positive control. The isotonic phosphate buffered saline (PBS) was used as negative control. All tests were performed in six replicates.

Minimum inhibitory concentrations (MICs) were performed in 96-well micro-plates (Eloff, 1998) using serial dilutions of Pp (500; 250; 125; 62.5; 31.25 µg/mL) and Pg (250; 125; 62.5; 31.25 µg/mL). Hundred microliters of Pp or Pg diluted in liquid culture medium and the tested microorganism suspensions (1.5 x 10^5 colony-forms unity CFU/well). After incubation (28°C for 24 h), the content of each well was sown in Petri dishes with agar culture medium. The Petri dishes were incubated for 48 hours at 28°C to account for the colony-forms unity (CFU). To indicate viable bacteria cells in the microplate, 10 µL of thiazolyl blue (tetrazolium salt 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) reagent were added to the microplate wells and incubated at 28°C for 1-3 h (Mosmann, 1983). The color change produced in reaction was measured in a spectrophotometer (540 nm) and the values were correlated to the viable bacteria cells in the microplate. MIC was measured as the lowest concentration necessary to inhibit growth of the tested pathogen. Minimum Bactericidal Concentration (MBC) was considered as the minimum concentration in which no growth was visually observed in Petri dishes with solid medium, with 99.99% of eradication of the initial inoculum (De Nova *et al.,* 2019). The concentration of the Pp and Pg that inhibited the growth of half of the inoculum was estimated as the inhibitory concentration 50 (IC_{50}) (Soothill *et al.,* 1992), represented as Log (inhibitor) versus normalized absorbance (%) (dose-response inhibition model). Streptomycin sulfate (500 µg/mL) and oxytetracycline hydrochloride (30 µg/mL) were used as positive control and isotonic phosphate buffered saline (PBS). All tests were performed in four replicates.

Effect of Hydroalcoholic crude extract from the fruit peel of the P. granatum on X. campestris pv. *campestris control in seeds*

Seeds of the *Eruca vesicaria* (L.) Cav. (Feltrin®, cultivated arugula variety, germination from 7 to 10 days) were purchased from a local market. Seeds were disinfected and coated with pathogenic bacteria according standard protocol (Kotan *et al.,* 2014). Seeds coated with pathogen were directly soaked in treatments consisting in: 1 - Pp suspension (500 µg/mL or 250 µg/mL) or 2 - association between Pp (500 µg/mL or 250 µg/mL) and antibiotic (streptomycin sulfate, 500 µg/ml) for 3 hours. The seeds were left to dry on sterile Whatman filter paper sheets overnight in laminar flow hood. The seeds were sown in plastic pots containing garden soil and sand (1:1) totaling thirty seeds per treatment (ten seeds/pot). Other part of the seeds was transferred to Petri dishes with Whatman paper filter placed on the bottom (moistened with 10 mL of sd. H_2O) totaling thirty seeds per treatment (ten seeds/plate). The percentage of germination and seedling emergence was determined 10-12 days after sowing. *E. vesicaria* seedlings were assessed 18 days after emergence to determine the appearance of symptoms of disease (Vicente and Holub, 2013) and survival rate. After this, the seedlings were removed from the substrate for assessment of the effect of the extract in growth promotion. Antibiotic (streptomycin sulfate at 500

µg/ml), disinfected seeds infected with pathogen, and sterilized seeds not infected with pathogen (healthy) were used as controls. All tests were performed in triplicates.

Anatomical analysis

Eruca vesicaria (L.) Cav. seedling samples leaves were preserved in fixative solution FAA (Formaldehyde, Glacial Acetic Acid, 95% EtOH) (Johansen, 1940). To prepare the samples, leaves were sectioned in transverse and paradermic sections using disposable razors. Leaf diaphanization was performed according to the standardized technique (Kraus and Arduin, 1997) and stained with safranin and Astra blue solutions, both at 0.5%. Semi-permanent slides were analyzed with optical microscope to visualize the adaxial epidermis, abaxial epidermis, palisade parenchyma, spongy parenchyma, stomatal density and stomatal morphology. Stomatal density (D) was calculated as D= number of stomata/(40x objective ocular area) (mm^2) (Abdulrahaman *et al.*, 2009). Public domain software ImageJ 1.43a version 64 (Schneider *et al.*, 2012) was used to obtain stomatal measures of the polar diameter (PD) (µm)**,** equatorial diameter (QD) (µm) and stomatal area (A) (μm^2) to each treatment. Stomatal functionality (FUN) was calculated as FUN=polar diameter (µm)/equatorial diameter (µm) of the stomata (De Castro *et al.,* 2009).

Statistical analysis

Results were expressed as the mean ± standard deviations. To determine difference between samples, one-way ANOVA followed by Tukey post hoc test and Student's t test were performed at $p<0.05$. Inhibitory concentration 50 (IC_{50}) was performed by Nonlinear Regression analysis (dose-response inhibition model) with 95% profile likelihood. All analyses were performed in GraphPad Prism® v. 8.0 software.

3. Results

Chemical characterization of the hydroalcoholic crude extract from the fruit peel of the P. granatum

The results demonstrated that Pp possessed 6.34 mg/g of ellagic acid and 0.83 g/g of total phenolic as tannic acid. HPLC chromatogram was performed focused on ellagic acid, and the fingerprint is presented in figure 1.

In vitro antimicrobial activity of the hydroalcoholic crude extract from the fruit peel of the P. granatum

Hydroalcoholic crude extract from the fruit peel of the *P. granatum* (Pp) was tested for its antimicrobial properties against phytopathogenic bacteria. Pp produced bacterial growth inhibition zones for all three investigated isolates (Table 1 and Fig. 2). The highest mean values of inhibition zones were verified for *R. solanacearum*, followed by *X. campestris* pv. *campestris* and *P. carotovorum* subsp. *carotovorum*. There was an increase of the inhibition zone produced as the increase of the *P. granatum* extract con-

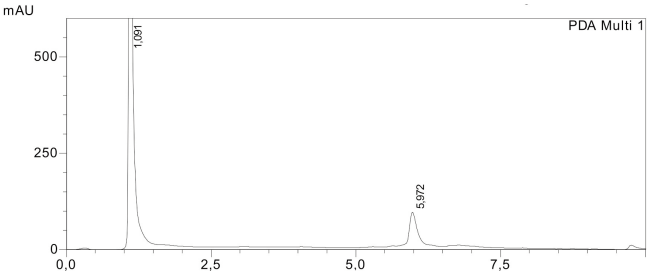

Fig. 1 - HPLC chromatogram of ellagic acid in *Punica granatum* extract (Pp). The extract was characterized considering the content of ellagic acid and the total phenolic as tannic acid by HPLC chromatography performed focused on ellagic acid. The mobile phase used for ellagic acid was methanol and acetic acid aqueous solution [2% - and elution gradient (0-7min, 20-72.5% v/v methanol, 7-7.5 min, 72.5-95% v/v methanol, 7.5-8.5 min. 95% v/v methanol, 8.5-9 min 95-20% v/v methanol, 9-10 min 20% v/v methanol)], flow rate of 1.0 mL min^{-1}, and oven temperature of 25°C -100 mm x 2.6 mm Shim pack ODS C18 column. The eluted samples were detected by UV detector at 254 nm.

Table 1 - *In vitro* inhibition zone produced by treatment with *Punica granatum* L. hydroalcoholic extract (Pp) against isolates of the phytopathogenic bacteria

Concentrations (mg mL^{-1})	Inhibition zones (mm)*		
	R. solanacearum	*X. campestris* pv. *campestris*	*P. carotovorum* subsp. *carotovorum*
Control#	42.83±1.4 a	45.03±0.6 f	35.17±0.75 j
100	35.33±1.2 b	28.83±0.7 g	22.67±0.51 k
50	31.17±2.1 b	26.33±0.8 g	20.00±0.89 k
25	25.67±1.8 c	23.0±1.0 g	16.67±1.03 l
12.5	22.17±2.6 c	19.5±0.5 g	10.17±5.11 m
6.25	16.0±2 d	15.67±1.3 h	ND
3.12	6.66±5.2 e	9.66±4.9 i	ND

Data were represented as mean ± standard deviation for six replications. ND= No detected of inhibition zone. # Control with antibiotics streptomycin sulfate for *P. carotovorum* subsp. *Carotovorum* and oxytetracyclin hydrochloride (Terramicin®) for *X. campestris* pv. *Campestris* and *R. solanacearum*.
The values followed by different letters (a-m) along each column are significantly different according to ANOVA followed by Tukey post hoc test ($p<0.05$).

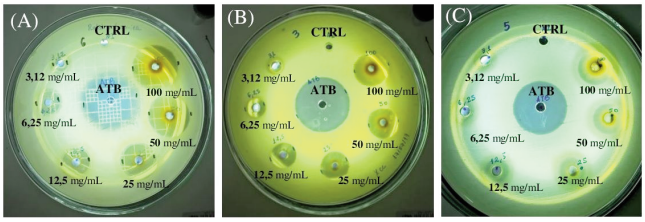

Fig. 2 - *In vitro* antimicrobial activity of *Punica granatum* L. hydroalcoholic extract (Pp) against isolates of phytopathogenic bacteria. Bacterial growth in Petri dishes with solid medium in agar-well diffusion assay. A) R. *solanacearum*; B) *X. campestris* pv. *campestris*; C) *P. carotovorum* subsp. *carotovorum*.
CTRL= negative control with PBS; ATB= positive control with antibiotic.

centration (dose-dependent effect) (Table 1).

In vitro antimicrobial activity through the microdilution test

Microdilution assays indicated susceptibility of the bacterial isolates *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris* to several concentrations of the Pp (Fig. 3 A-E and 4 A-E) and Pg (Fig. 3 B-E and 4 B-E). Were made tests with different concentrations of the Pp (500 to 31.25 µg/mL) to verify cell viability of the bacteria in comparision with negative control and antibiotic. Bacteria in the negative control remained with high celular viability. In the groups treated with Pp in the highest concentrations (500 and 125 µg/mL) there was a reduction of the cell viability of the both bacteria in relation to the negative control (Fig. 3 A-C and 4 A-C). The antimicrobial effect of the Pp in concentrations of 500 and 250 µg/mL for *X. campestris* pv. *campestris* was similar to antibiotic and different of the negative control (Fig. 4 A-B). Concerning to *P. carotovorum* subsp. *carotovorum*, the antimicrobial effect of the Pp in concentration of 500 µg/mL was similar to antibiotic and different of the negative control (Fig. 3 A).The smallest concentrations of the Pp (62.5 and 31.25 µg/mL) did not produce any antimicrobial effect in cell viability

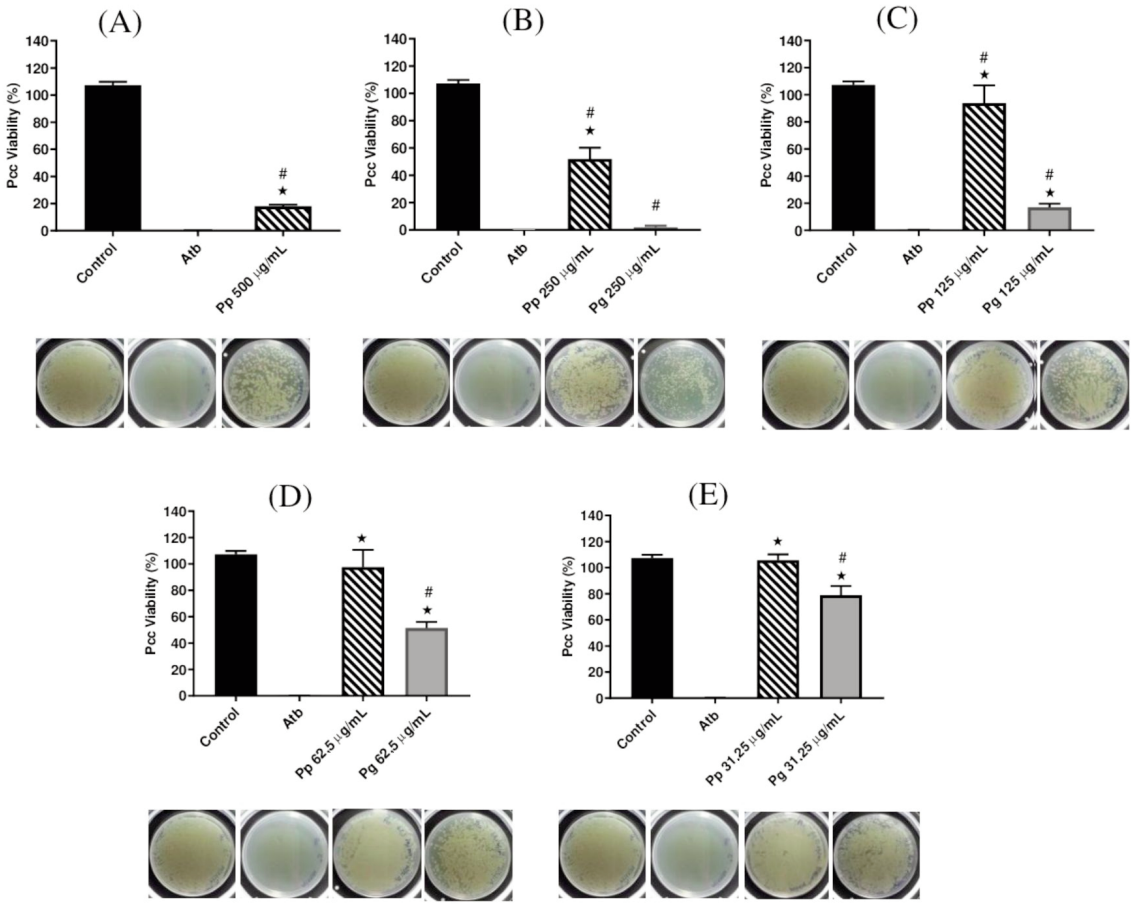

Fig. 3 - *In vitro* inhibition rates of the *P. carotovorum* subsp. *carotovorum* cell viability (%) for treatments with *Punica granatum* L. hydroalcoholic extract (Pp) and Punicalagin compound (Pg). Each graph was accompanied of bacterial growth in Petri dishes with agar solid medium. (*) significant difference between treatments (Pp or Pg) and antibiotic (Atb); (#) significant difference between treatments (Pp or Pg) and negative control (Control) ($p<0.05$, ANOVA followed by Tukey post hoc test). Each bar represents mean ± SD.

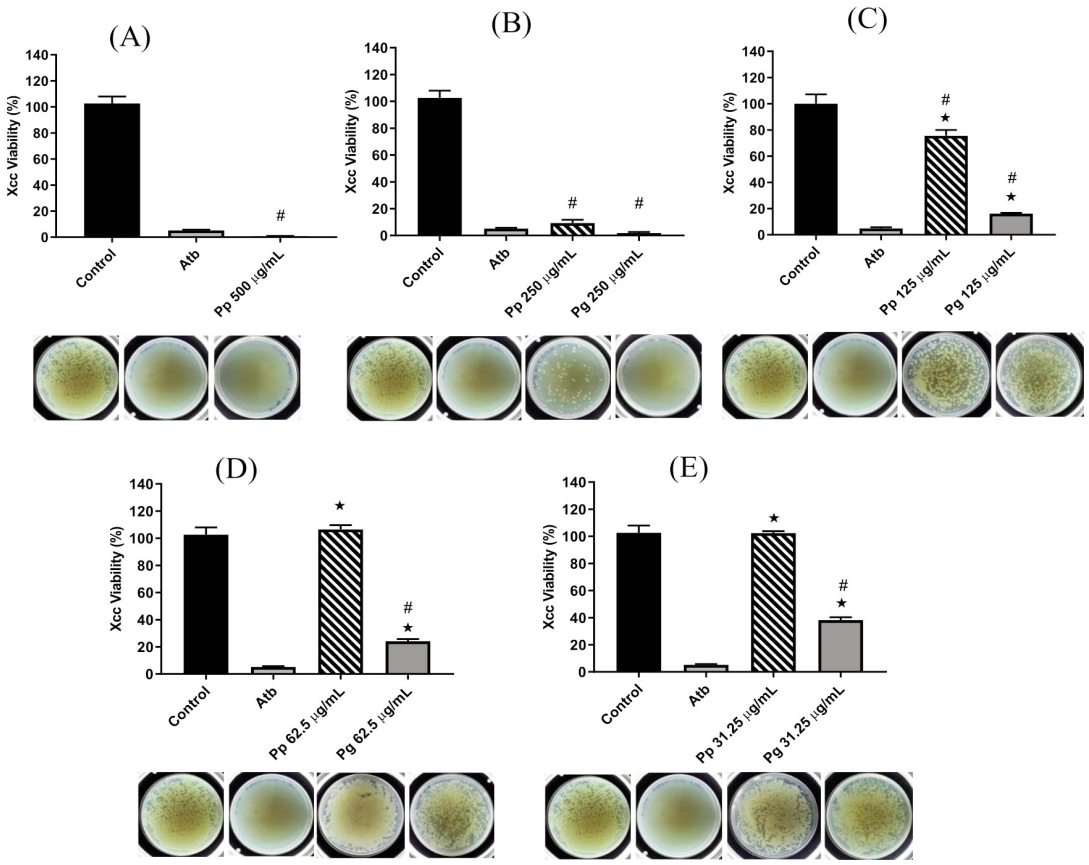

Fig. 4 - *In vitro* inhibition rates of *X. campestris* pv. *campestris* cell viability (%) for treatments with *Punica granatum* L. hydroalcoholic extract (Pp) and Punicalagin compound (Pg). Each graph was accompanied of bacterial growth in Petri dishes with agar solid medium. (*) significant difference between treatments (Pp or Pg) and antibiotic (Atb); (#) significant difference between treatments (Pp or Pg) and negative control (Control) ($p<0.05$, ANOVA followed by Tukey post hoc test). Each bar represents mean ± SD.

for these both pathogens (Fig. 3 D-E and 4 D-E).

Antimicrobial activity of the Pg against the investigated pathogens was higher than action of the Pp. The lowest concentration of the Pg that inhibits bacterial growth (MIC) to both *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris* was 31.25 µg/mL (Fig. 3E and 4E). Punicalagin compound at the highest tested concentration (250 µg/mL) showed antibiotic-like antimicrobial activity, in terms of the cellular viability of the both pathogens (Fig. 3B and 4B). The observation of bacterial growth in culture plates with agar medium indicated that Pg in this concentration (250 µg/mL) may present bacteriostatic action for *P. carotovorum* subsp. *carotovorum* (Fig. 3B) or bactericidal action to *X. campestris* pv. *campestris* (Fig. 4B).

Regarding to the concentration that inhibits 50% of bacterial inoculum (IC_{50}) Pp presented a concentration of 212 µg/mL to *P. carotovorum* subsp. *carotovorum* (Fig. 5A) and 154.6 µg/mL to *X. campestris* pv. *campestris* (Fig. 5B). The concentration of the Pg compound that inhibits 50% of bacterial inoculum (IC_{50}) was 58.96 µg/mL to *P. carotovorum* subsp.

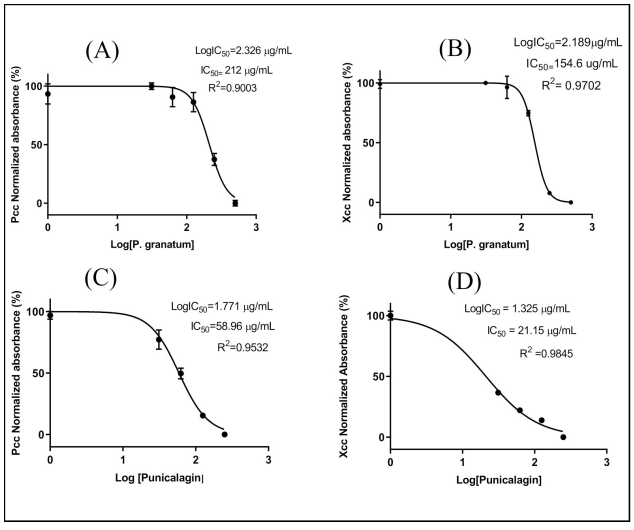

Fig. 5 - Inhibitory concentration 50 (IC50) of the treatments with *Punica granatum* L. hydroalcoholic extract (Pp) and Punicalagin compound (Pg) against isolates of the phytopathogenic bacteria. A) and B) represent inhibitory effect of the treatment with Pp on *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris*. C) and D) represent inhibitory effect of the treatment with Pg on *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris* by the Nonlinear Regression (dose-response inhibition model) with 95% profile likelihood.

carotovorum (Fig. 5C) and 21.15 µg/mL to *X. campestris* pv. *campestris* (Fig. 5D).

In vivo antimicrobial and biostimulant activity of the hydroalcoholic crude extract from the fruit peel of the P. granatum in E. vesicaria seeds infected by X. campestris pv. campestris

The most effective concentrations of the Pp in microdilution assays (500 µg/mL and 250 µg/mL) were tested for the control of the *X. campestris* pv. *campestris* in *E. vesicaria* seeds. Infected and untreated seeds (negative control) presented a lower emergence percentage compared to healthy seeds. On the other hand, treatment of infected seeds with Pp (500 µg/mL) promoted an increase of the 15% in the percentage of emergence in relation to the negative control (Table 2).

In addition, *E. vesicaria* seedlings treated with Pp did not develop main symptom of black rot disease caused by *X. campestris* pv. *campestris* (the "V" chlorotic lesion in the margin of the leaflet). This symptom was verified in seedlings of infected and untreated seeds. There was no phytotoxic effect of the Pp in seedlings development. Seedlings of the *E. vesicaria* treated with highest concentration of the Pp (500 µg/mL) showed a biggest growth length of radicle (Fig. 6). Treatment with streptomycin sulfate crude or associated with Pp resulted in seedlings with

Table 2 - *In vivo* activity of the *Punica granatum* L. hydroalcoholic extract (Pp) in germination and emergency of the *Eruca vesicaria* L. (Cav.) seeds infected with *X. campestris* pv. *Campestris*

Treatment*	Concentration (µg/mL)	Germination (%)	Emergency (%)
Health#	-	93.3±5.1 a	80±0.0 b
Ctrl-	-	93.3±5.1 a	45±5.7 c
Ctrl+	500	93.3±10.33 a	65±5.7 d
Pp	500	100±0.0 a	60±11.55 d
	250	93.3±5.1 a	45±5.7 d
Pp + Atb	500 + 500	93.3±5.1 a	60±11.55 d
	250 + 500	86.6±10.3 a	60±11.55 d

Health= seeds uninfected with pathogen;*Ctrl- = negative control (seeds infected and untreated); Ctrl+ = positive control (seeds infected treated with streptomycin sulfate); Pp= seeds infected treated with *Punica granatum* L. hydroalcoholic extract (Pp) at concentrations of 500 µg/mL and 250 µg/mL; Pp + Atb= association between Pp (500 µg/mL or 250 µg/mL) and antibiotic streptomycin sulfate (500 µg/mL). In each column, values followed by different letters (a-d) are significantly different according to ANOVA followed by Tukey post hoc test ($p<0.05$).

Fig. 6 - Growth length of radicle of the *Eruca vesicaria* L. (Cav.) seedlings treated with *Punica granatum* L. hydroalcoholic extract (Pp). (A) seedlings of healthy seeds; (B) seedlings of infected with *X. campestris* pv. campestris and untreated seeds; (C) seedlings of seeds infected and treated with streptomycin sulfate; (D) seedlings of seeds infected and treated with Pp (500 µg/mL); (E) seedlings of seeds infected and treated with Pp (250 µg/mL); (F) seedlings of seeds infected and treated with association between Pp (500 µg/mL) and streptomycin sulfate.

Fig. 7 - Appearance of *Eruca vesicaria* L. (Cav.) seedlings germinated in Petri dishes and in pots with different treatments. Healthy seeds – uninfected seeds; Seeds with antibiotic: seeds infected with *X. campestris* pv. *campestris* and treated with streptomycin sulfate; Seeds with pomegranate extract: seeds infected and treated with *Punica granatum* L. hydroalcoholic extract (Pp) (500 µg/mL).

chlorosis symptom (yellowish leaves) (Fig. 7).

The results of this study showed different seedling survival rates of the *E. vesicaria* according to each treatment (Fig. 8A-D). Infected and untreated seedlings (negative control) showed an abrupt drop in the percentage of survival at the 7th day after emergence. Seedlings treated with Pp at the highest concentration (500 µg/mL) showed slowly decrease

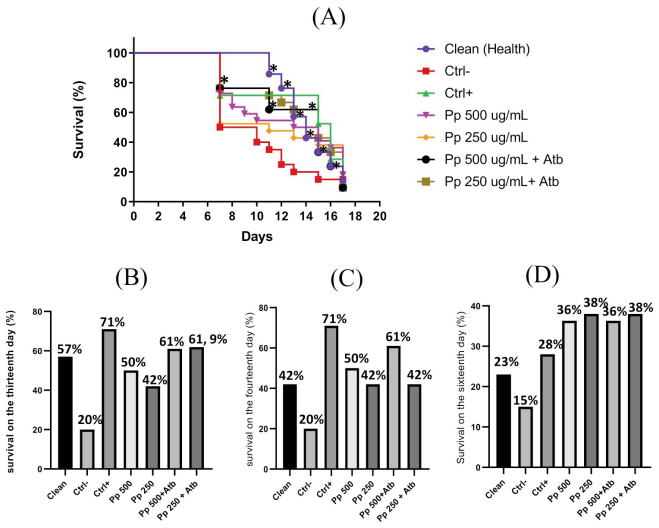

Fig. 8 - *In vivo* effect of the treatment with *Punica granatum* L. hydroalcoholic extract (Pp) in survival rates of *Eruca vesicaria* L. (Cav.) seedlings germinated in pots. Clean (health)= seeds uninfected with *X. campestris* pv. *campestris*; Ctrl- = infected and untrated seeds; Ctrl+= seeds infected and treated with streptomycin sulfate; Pp 500 µg/mL and Pp 250 µg/mL= Seeds infected and treated with *Punica granatum* L. hydroalcoholic extract (Pp); Pp 500 µg/mL + Atb or Pp 250 µg/mL + Atb= seeds infected and treated with association between *Punica granatum* L. hydroalcoholic extract (Pp) and streptomycin sulfate. A) total survival rates; B) survival rates in thirteenth day; C) survival rates in fourteenth day; D) survival rates in sixteenth day. Means with (*) are different from the negative control by the Student's t test at p<0.05.

in the percentage of survival in the same period (Fig. 8A). On the 13th day after emergence, the survival rates of the seedlings treated with Pp (500 µg/mL and 250 µg/mL) were 50% and 42%, respectively, versus 20% of the negative control (Fig. 8B). Association between Pp (500 µg/mL and 250 µg/mL) and antibiotic also promoted higher survival rates in relation to negative control in the same period (61% and 61.9%, respectively) (Fig. 8B). After 16 days of seedling emergence there were similar rates for treatment with Pp (500 µg/mL and 250 µg/mL) and treatment with association between Pp and antibiotic. These percentages remained high (36%-38%) in relation to negative control (15%) (Fig. 8D).

Anatomical analyses of *E. vesicaria* seedling leaves indicated differentiation of mesophilic structures, especially in relation to the palisade parenchyma (Fig. 9A-F). Uninfected (healthy) seedlings presented well-preserved anatomical structures (Fig. 9A). Seedlings of the infected and untreated seeds group (negative control) showed some alterations in mesophilic tissue, especially in relation to incomplete differentiation of palisade parenchyma, when compared to healthy plants (Fig. 9B). Seedlings in the group previously treated with streptomycin sulfate presented altered palisade parenchyma cells in a more rounded shape (Fig. 9C). Seedlings treated with Pp at a concentration of 500 µg/mL showed clear differentiation of mesophilic structures, with well-structured palisade parenchyma (Fig. 9D). Seedlings treated with Pp at a concentration of 250 µg/mL did not present clear differentiation of mesophilic elements (Fig. 9E).

Appearance of stomata of seedlings in different treatments with Pp was represented in figure 10 (A-F). From these images, the mean values of the fol-

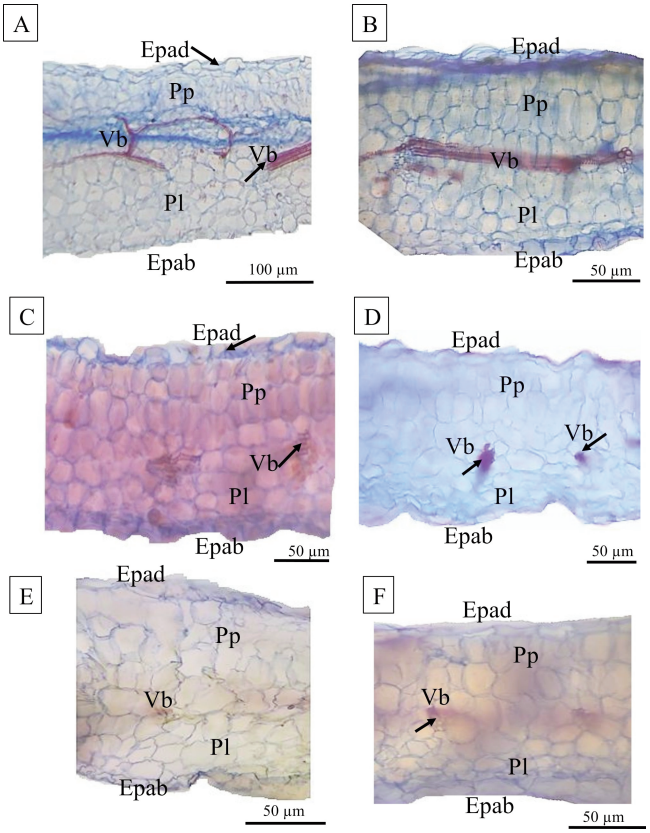

Fig. 9 - Cross sections of the mesophyl of *Eruca vesicaria* L. (Cav.) seedlings infected with the *X. campestris* pv. *campestris* submitted to different treatments. A) Uninfected seedlings; B) seedlings infected with *X. campestris* pv. *campestris* and untrated; C) seedlings infected and treated with streptomycin sulfate; D) Seedlings infected and treated with *Punica granatum* L. hydroalcoholic extract (Pp) (500 µg/mL); E) Seedlings infected and treated with *P. granatum* L. hydroalcoholic extract (Pp) (250 µg/mL); F) Seedlings infected and treated with association between *P. granatum* L. hydroalcoholic extract (Pp) (500 µg/mL) and streptomycin sulfate.
Epad= adaxial epidermis; Abed= abaxial epidermis; Lp= lacunous parenchyma; Pp= palisade parenchyma; Vb= vascular bundle.

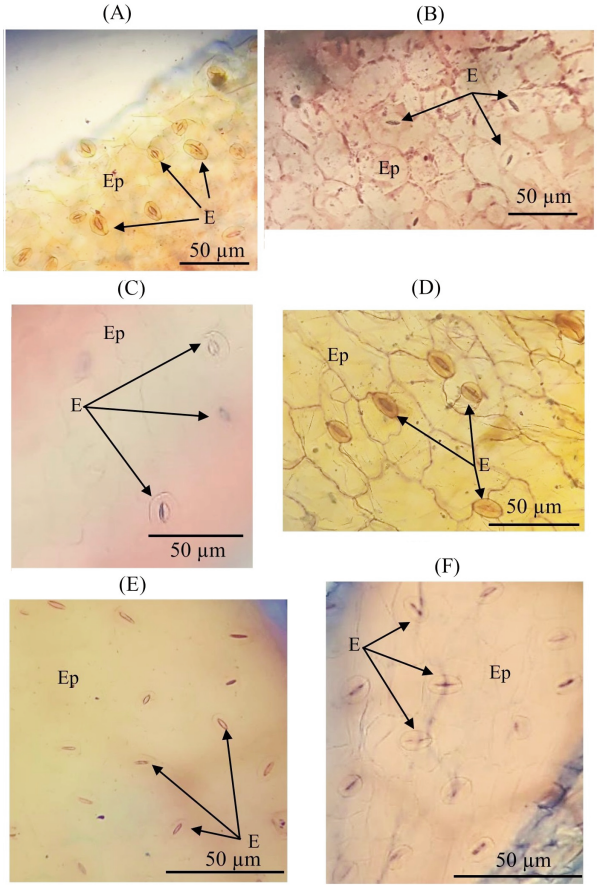

Fig. 10 - Paradermic sections of the leaf blade of *Eruca vesicaria* L. (Cav.) seedlings submitted to different treatments. A) Uninfected seeds; B) seeds infected with *X. campestris* pv. *campestris* and untrated; C) seeds infected and treated with streptomycin sulfate; D) seeds infected and treated with *Punica granatum* L. hydroalcoholic extract (Pp) at concentration of 500 µg/mL; E) seeds infected and treated with *P. granatum* L. hydroalcoholic extract (Pp) at concentration of 250 µg/mL; F) seeds infected and treated with association between *P. granatum* L. hydroalcoholic extract (Pp) (500 µg/mL) and streptomycin sulfate. St= stomata; Ep= epidermis.

lowing parameters were taken: polar diameter (PD), equatorial diameter (QD), stomatal functionality (FUN), area (A) and stomatal density (SD) (Table 3). Seedlings of the group treated with Pp (500 µg/mL) showed highest values of PD, FUN and A (µm²) in comparison to negative and positive control (Table 3). Stomatal functionality of the group treated with Pp (500 µg/mL) was similar to that of healthy seedlings. The highest mean of SD was verified for the group treated with Pp at a concentration of 250 µg/mL, being statistically similar to the clean group. Seedlings of the infected and untreated group showed smallest values of PD, FUN and SD. Small values of PD and FUN were observed too in seedlings treated with antibiotic (Table 3).

4. Discussion and Conclusions

In the present study, the effective antimicrobial action of the *P. granatum* fruit peel extract and its isolated punicalagin compound on phytopathogenic bacteria (*R. solanacearum*, *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris*), listed among the ten most important species in scientific and economic aspects worldwide (Mansfield *et al.,* 2012), was shown . The wide zones of inhibition of bacterial growth in agar-diffusion tests and marked reduction in the percentage of the cell viability in broth microdilution assays indicate high potential of pomegranate extract in the control of these phytopathogens.

Pomegranate peel has substantial amounts of phenolic compounds, such as hydrolysable tannins (punicalin, punicalagin, ellagic acid, and gallic acid), flavonoids (anthocyanins and catechins), and nutri-

Table 3 - Stomatal measures in paradermic sections of leaves of the *Eruca vesicaria* L. (Cav.) submitted to different treatments

Treatments	Stomatal measures *				
	PD	QD	FUN	A (µm²)	SD (mm²)
Health	25.2±4.5	14.9±1.07 bcdef	1.71±0.3 c	302.5±21.3 def	77.7±9.8 bcdf
Ctrl-	22.9±2.3 d	18±1.2 a	1.28±0.1 ad	348.5±36.8 df	29.3±13.7 acdef
Ctrl+	21.5±2.9 df	18.2±0.8 a	1.18±0.1 ade	270±19.6 d	46.9±5.4 abed
Pp 500 µg/ml	29.6±4 bcef	17.6±1.9 a	1.69±0.2 bcef	483±41.1 abce	49.1±7.6 ab
Pp 250 µg/ml	22.9±1.4 d	16.7±1 a	1.37±0.01 cd	335±19.7 ad	87.2±15.8 bcd
Pp 500 µg/ml +Atb	26±2.3 c	19.4±3.6 a	1.36±0.1 cd	449.8±49.2 ab	51.3±5.7 ab

Health= uninfected seedlings; Ctrl- = infected and untreated seedlings (negative control); Ctrl+ = seedlings infected and treated with streptomycin sulfate (positive control); Pp 500 µg/mL and Pp 250 µg/mL= seedlings infected and treated with *P. granatum* L. hydroalcoholic extract (Pp) at concentration of 500 µg/mL or 250 µg/mL; Pp 500 µg/mL + Atb= seedlings infected treated with *P. granatum* L. hydroalcoholic extract (Pp) at concentration of 500 µg/mL in association with antibiotic streptomycin sulfate (500 µg/mL).

* Data were represented as mean ± standard deviation for three replications. PD= polar diameter (µm); QD= equatorial diameter (µm); FUN=- stomatal functionality; A= stomatal area; SD= stomatal density (mm2). In each column, values followed by different letters (a-f) are significantly different according to Student's t test ($p<0.05$) where: a- comparison with health; b-comparison with Ctrl-; c-comparison with Ctrl+; d-comparison with Pp 500 µg/ml; e-comparison with Pp 250 µg/ml; f-comparison with Pp 500 µg/ml +ATB.

ents, which are responsible for its biological activity (Magangana *et al.,* 2020). The fruit peel has high antioxidant and antimicrobial activities and may be used as an excellent natural additive for food preservation and for quality enhancement. The health-promoting benefits of pomegranate peel have prompted the food industry to focus on pomegranate-peel-containing food preparations, which include nutraceuticals, phenolic-enriched diets, and food supplements (Opara *et al.,* 2009; Fawole *et al.,* 2012).

The Pp showed both bacteriostatic (*P. carotovorum* subsp*. carotovorum,*) and bactericidal effect (*X. campestris* pv*. campestris*). Bactericidal effect is desirable in order to inhibit the emergence of resistant bacterial strains and toxicity (Soberón *et al.,* 2014). The study results support literature data on the antimicrobial action of the ethanolic *P. granatum* fruit peel extract against phytopathogenic bacteria *Pseudomonas syringae* pv*. tomato*, the cause of bacterial spot disease in tomatoes, with bacterial growth inhibition zones of 5-26 mm and dose-dependent effect (Quattrucci *et al.,* 2013). Additionally, the results agreement with studies that reported antibacterial action of the methanolic pomegranate peel extract against *R. solanacearum*, with growth inhibition zone of the 13.9 mm (50 mg/mL), and ethyl acetate extract against *R. solanacearum*, *P. carotovorum* subsp. *carotovorum* and *X. gardneri* with inhibition zone of 8.5-22.75 mm (concentrations of 25-200 mg/mL) (Farag *et al.,* 2015; Khaleel *et al.,* 2016). Studies with extracts from other parts of the plant (leaf and seed) have shown more discrete antimicrobial activity on *R. solanacearum* and *X. campestris* (Hassan *et al.,* 2009; Uma *et al.,* 2012).

Antimicrobial activity of the *P. granatum* fruit peel extract against Gram-negative and Gram-positive bacteria has been correlated with the presence of polyphenolic compounds in it, mainly punicalagin (Gullon *et al.,* 2016). A relatively high amount of polyphenols (867 mg/g) was detected in a pomegranate peel extract preparation, especially the ellagitannin punicalagin (296 mg/g), with antimicrobial action of the extract on isolates of *S. aureus, Escherichia coli, Aspergillus niger* and *Saccharomyces cerevisiae* (Ibrahium, 2010). The antimicrobial mechanism of action of polyphenols seems to be related to the direct action of these compounds on the bacterial cell wall by formation of complexes with wall proteins, causing lysis (Akhtar *et al.,* 2015). The interaction of these compounds with sulfhydryl groups of extracellular microbial proteins results in inhibition of protein activity (Dey *et al.,* 2012).

The impressive antimicrobial action of punicalagin on *P. carotovorum* subsp. *carotovorum* and *X. campestris* pv. *campestris* verified in the present study suggests that this molecule may be an essential component in the biological activity of *P. granatum* fruit peel extract against phytopathogenic bacteria. Several studies prove antimicrobial activity of the punicalagin compound against isolates of clinical importance such as *Staphylococcus aureus* (MIC 250 µg/mL) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Xu *et al.,* 2017; Mun *et al.,* 2018). According to Xu *et al.* (2017) punicalagin compound has direct action on cell membrane disruption, increased K^+ ion flow and inhibition of biofilm formation in *S. aureus*. To the best of our knowledge, this is the first report of the antibacterial activities of the isolated compound punicalagin against plant pathogenic bacteria. Further investigations may elucidate the mechanism of action of this compound on phytopathogenic bacteria.

Data from the *in vivo* assays of this study indicated that Pp is effective in control of *X. campestris* pv. *campestris* in *E. sativa* seeds. Black rot infection causes tissue necrosis, premature leaf fall, atrophied growth and death of young plants (Vicente and Holub, 2013). In the present study, treatment with Pp in the highest concentration reduced incidence of disease symptoms and promoted a high survival rates of seedlings in comparison to infected and untreated group. Additionally, the association between Pp and antibiotic resulted in percentages of seedling survival above that observed in treatment with antibiotic alone (16^{th} day of observation), indicating probable synergistic interaction between treatments. *P. granatum* extract produced no harmful effect on germination, emergence or seedling development of the *E. sativa*. This is in agreement with a study that proved the effective action of treatment with natural plant extract (*Origanum onites*) in the control of *Clavibacter michiganensis* ssp*. michiganensis*, *Xamthomonas axonopodies* pv*. vesicatoria* and *Xanthomonas campestris pv. vitians* in seeds of tomato and lettuce, without affecting seedling germination and growth (Kotan *et al.,* 2014). Treatment of the seeds with hot water (50°C for 20-30 min) has been the most effective treatment for seedborn blackrot control. However, treatments do not always eliminate 100% of the bacteria and may adversely affect seed germination and vigor (Celetti and Callow, 2002). Natural plant extracts, like pomegranate fruit

peel extract, may represent a good alternative to control of seed born phythopatogens ensuring seed viability after treatment.

Treatment of *E. vesicaria* seeds with streptomycin sulfate, despite being effective in controlling *X. campestris* pv. *campestris*, demonstrated toxic effect for seedlings, evidenced by the yellowish aspect of the leaves (chlorosis). This result corroborates the findings of Napoles *et al.* (1991) for treatment of *Brassica oleracea* seeds with the same antibiotic (500 ppm for 1 h). The streptomycin is associated with several phytotoxic effects like blocking chlorophyll synthesis, especially in younger leaves, inhibition of methionine and phosphate absorption, production of photosynthetic process changes and enzymatic inhibition (Falkiner, 1990). In addition, treatment of seeds with streptomycin resulted in severe reduction in the ratio between the polar and equatorial diameters (stomatal functionality). However, treatment with Pp (500 µg/ml) keep values of stomatal functionality (FUN) similar to the healthy seedlings. The highest ratio of FUN indicates stomata with more elliptic morphology, a mechanism of the drought tolerant plants to keep the water present in its interior at a maximum as a response to its hydric state (Melo *et al*., 2014).

Treatments with Pp indicated antagonistic values of stomatal density (SD) and stomatal area (A). The parameters SD and A can directly affect mechanisms such as photosynthesis, transpiration and efficient water use in plants (Lawson and Blatt, 2014). The increase in SD coupled with reduction in A may result in the optimization of gas exchange (Franks *et al.,* 2009). This pattern was observed in seedlings treated with Pp at a concentration of 250 µg/ml and similarly in the health group. On the other hand, the reduction in SD may represent a more conservative water use (Bertolino *et al.,* 2019). This pattern was verified in seedlings treated with Pp at a concentration of 500 µg/ml. Since there is no damage to CO_2 fixation or plant cooling, this reduction in water loss can be advantageous in environments with low water availability (Bertolino *et al.,* 2019). Thus, the Pp can influence both pathogen control in seeds and the physiological characteristics of the plant through changes in stomatal patterns.

In conclusion, hydroalcoholic crude extract from the fruit peel of the *P. granatum* (Pp) demonstrated high potential for control of the phytopathogenic bacteria *R. solanacearum*, *X. campestris* pv. *campestris* and *P. carotovorum* subsp. *carotovorum*. Punicalagin compound potentiated antimicrobial activity on these pathogens, corroborating studies that relate the abundance of the phenolic compounds (flavonoids and tannins) with the antibacterial activity of pomegranate fruit peel. Pp extract was effective to control seedborn pathogen *X. campestris* pv. *campestris* in seeds of the *E. vesicaria* and promoted several beneficial effects to seedlings with no phytotoxic effect. Moreover, association between Pp and antibiotic indicated probable synergistic interaction between treatments (16th day of observation) potencializing the seedling survival over the observed in relation of the antibiotic utilizing in isolated way.

The investigation of antimicrobial activity of the Pp and isolate compounds like Punicalagin represents a promising path regarding the biotechnological development of botanical pesticides that ensure quality and safe of the food crop production.

Acknowledgements

The Coordination for the Improvement of Higher Education Personnel (CAPES) primarily funded this work - [Finance code 001].

This work was supported by the Foundation for Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão (FAPEMA) - Brazil.

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Adv. Hort. Sci., 2021 35(3): 233-241 DOI: 10.36253/ahsc-9923

AHS
Advances in Horticultural Science

Effect of growth temperature levels on photosynthetic ability and fruit quality of 'KU-PP2', a new low-chill peach cultivar

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Key words: forcing culture, high temperature, protected culture, *Prunus persica*, stress response.

Abstract: Temperature is a crucial factor in growing plants in a forcing system. Our goal was to introduce low-chill peach cultivars into a forcing culture for early-season peach production with high fruit quality. However, the effects of growth temperature on plant growth and fruit quality during fruit development of the 'KU-PP2' peach cultivar have not yet been evaluated. 'KU-PP2' trees were grown in containers and transferred to phytotrons after fruit set in April 2019. The air temperature was set at 20, 25, and 30°C until harvest. Photosynthetic ability, leaf characteristics, and fruit quality under each treatment were determined. Long exposure to lower growth temperatures did not cause a change in leaf characteristics or a reduction in photosynthetic ability and fruit quality in the 'KU-PP2' peach cultivar. In contrast, the 30°C was found to be associated with a decrease in leaf size and thickness, stomatal density, photosynthesis, chlorophyll content, and fruit size. Conversely, the high-temperature condition enhanced coloration of the fruit peel and hastened the harvesting period, compared with the lower-temperature treatments. These results indicated that long-term exposure to the moderately high temperature of 30°C negatively affected plant growth and fruit productivity through changed leaf characteristics and a disrupted photosynthesis.

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Citation:
SIKHANDAKASMITA P., KATAOKA I., OGATA T., MOCHIOKA R., BEPPU K., 2021 - *Effect of growth temperature levels on photosynthetic ability and fruit quality of 'KU-PP2', a new low-chill peach cultivar.* - Adv. Hort. Sci., 35(3): 233-241

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 19 October 2020
Accepted for publication 10 June 2021

1. Introduction

Air temperature is a crucial factor that affects fruit production. Excessive high temperatures disrupt normal plant functions such as carbon assimilation, respiration, fertilization, cell differentiation, and fruit maturation (Cui *et al*., 2006; Efeoglu and Terzioglu, 2009; Lin-Wang, 2011; Hao *et al*., 2019). Previous report indicated that chlorophyll (Chl) *a* content, total Chl content, and the Chl *a/b* ratio in soybeans, which were grown under high temperatures (38/28°C), decreased 7, 3, and 18%, respectively (Hasanuzzaman *et al*., 2013). Additionally, Sugiura *et al*.

(2003) showed that higher temperatures significantly change the fruit quality of apple. A reduction in acid concentration and softening of fruit flesh were observed resulting from exposure to high temperatures during fruit development. The effect of elevated temperatures on plants differs depending on the stages of development and timescale. Continuing heat stress can lead to slowing of growth and development and inducing an imbalance in carbohydrate metabolism between photosynthesis and respiration. As a result, carbohydrate reserves decline, leading of yield loss, and possibly plant death (Hall, 1992; Wahid *et al*., 2007).

Photosynthesis comprises a few principal components that are highly sensitive to temperature: photosynthetic pigments, electron transport chain, Photosystem I (PS I), and Photosystem II (PS II). The decline in photosynthesis under high-temperature conditions results from inhibition of the redox reaction and metabolic pathways occurring in PS I, PS II, the cytochrome complex, and photosynthetic enzyme activities (Taiz and Zeiger, 2006). Moreover, elevated temperatures can also affect photosynthesis via physical processes. Previous studies have shown that heat stress is involved with leaf water status, leaf gas exchange, and stomatal conductance (*gsw*) caused by changes in hydraulic conductance (Fredeen and Sage, 1999; Greer and Weedon, 2012). Under high-temperature conditions, intercellular CO_2 concentration in leaves frequently declines because of stomatal closure and reduced CO_2 uptake and transport, leading to impaired photosynthetic CO_2 assimilation (Centritto *et al*., 2001).

'KU-PP2' is a new yellow flesh peach with a low-chilling requirement that produce excellent yield and high fruit quality. It was bred and released in 2016 for use in subtropical regions and particularly for use in forcing culture system to expand the harvesting season of fresh peach (Manabe *et al*., 2015). Understanding the effect of growth temperatures on 'KU-PP2' peach trees is crucial for optimizing plant growth, physiological functioning, and increasing productivity. The effect of chilling accumulation and heating temperatures on bud burst and flowering of 'KU-PP2' have been clarified. However, the influence of temperature during fruit development on low-chill peach cultivars has not been elucidated. Previous studies on Japanese high-chill peach cultivars indicated that high temperatures dramatically hasten fruit growth and the onset of fruit maturation (Sugiura *et al*., 2003; Hayama *et al*., 2007). In addition, optimal heating could save energy costs for plant production in heated plastic houses. Therefore, the aim of this study was to investigate the effect of growth temperature during fruit development on plant physiology and to determine the optimal growth temperature for plant growth, which can enhance fruit quality of the low-chill peaches under controlled conditions. Additionally, the knowledge gained could be used to design a heating program and cultivation management practices for growing the low-chill peach trees in plastic houses.

2. Materials and Methods

Plant materials

This experiment was conducted at the research field of the Faculty of Agriculture, Kagawa University, which is located in southwest Japan. Six healthy and uniform of seven-year-old 'KU-PP2' peach trees were selected for this study. All plants were grafted onto 'Tsukuba 1 Gou' peach rootstock and planted in containers. 'KU-PP2' flowers were hand-pollinated with fresh pollen from another 'KU-PP2' tree. Three weeks after pollination, two plants were transferred to each temperature regimes and the fruits were thinned by hand to 6-7 fruits per tree. The air temperature in the phytotrons was set at 20, 25, and 30°C during the experimental period from 13 April to 8 July 2019. Cultural practices and fertilization were performed according to standard peach growing practices in Japan (Sugiura *et al*., 2003).

Leaf morphology and anatomy observation

Leaf length and width were measured for five mature leaves per tree for each treatment using a digital caliper at the end of the experiment. Leaf width was measured across the widest part of the leaf. Five fully expanded leaves were collected and weighed immediately to determine fresh weight. These leaves were dried in a hot air oven at 80°C and weighed after 72 h of drying to determine their dry weight (Fanourakis *et al*., 2017). Leaf dry matter (DM) was calculated as the ratio between dry mass and fresh mass. DM was expressed as the percentage of fresh weight For anatomical analysis, five leaf samples from each plant were collected and preserved in formalin-acetic acid-alcohol (FAA; formaldehyde 1:acetic acid 1: 99.5% ethanol 9:deionized water 9) solution. Cross-sections were made using a rotary microtome at a thickness of 5 µm. The cross-sections

of samples were observed and photographed using a light microscope equipped with a microscope camera (Olympus DP-25, Olympus Co. Ltd., Japan). The following anatomical characteristics were measured: the thickness of the adaxial and abaxial epidermis, spongy mesophyll, and palisade cells, as well as the number of stomata per square millimeter.

Evaluations of chlorophyll content and SPAD value

The Chl content and SPAD value of five mature leaves from each tree were analyzed during the harvesting period. SPAD values were measured using a portable chlorophyll meter (SPAD-502, Minolta, Japan). Chlorophyll in the same leaves was analyzed as described by Lichtenthaler and Wellburn (1983). Leaf disks (2.5 cm^2 per disk) were homogenized with 10 mL of cold 95% acetone and incubated at 4°C in darkness for 3 h. These mixtures were centrifuged at 3,500 rpm for 10 min. After centrifugation, absorbance of the supernatants was determined using the spectrophotometer. The optical density for the blank and the mixtures were measured at 645 and 663 nm, respectively. These absorbance values were used to calculate Chl *a*, Chl *b*, and total chlorophyll (Chl *a+b*) and expressed as mg L^{-1}.

Leaf gas exchange measurement

Photosynthetic gas exchange was measured using a Portable Photosynthesis System (LI-6800; LI-COR Biosciences, Lincoln, NE, USA) from 9:00 to 12:00. The rate of net CO_2 assimilation, stomatal conductance, transpiration, and intercellular CO_2 concentration were measured weekly until the end of the experiment. Ten newest fully expanded leaves, which were outside of the canopy and fully exposed to sunlight, were randomly selected and used for the measurements. The reference CO_2 concentration and flow rate inside the chamber were maintained at 400 µmol mol^{-1} and 800 µmol m^{-2} s^{-1}, respectively. Photosynthetically active radiation (PAR) was set to 1,200 µmol s^{-1}. The chamber temperature was comparable to the growth temperatures, and relative humidity (RH) was kept at 60% (Marchi *et al*., 2008). The data were recorded at a steady state, in which gas exchange parameters were stable.

Fruit quality assessment

Five fruits per treatment were collected on the commercial harvest date for phytochemical analysis. After the harvest, all fruits were immediately transferred to the laboratory and weighed. Flesh firmness and total soluble solids (TSS) were measured from two opposite fruit cheeks. Flesh firmness was determined using a manual penetrometer with a 4.5-mm tip. TSS was measured using a digital refractometer (PR-101α; Atago Co. Ltd., Japan) and were expressed as degree Brix (°Brix). Titratable acidity (g L^{-1} of malic acid) was determined by titrating fruit juices with 0.05 mol L^{-1} of sodium hydroxide (NaOH) using Acidity Titrator (TA-72; DKK-TOA Co. Ltd., Japan). The fruit development period was calculated as the days from full bloom to first commercial harvest. Fruit coloring was estimated visually according to a scale from 1 (none) to 9 (hiding ground color) using ECPGR priority descriptors for peach (UPOV, 2010). The fruit coloring was expressed as the percentage of over color extent.

Statistical analysis

All data from each treatment were subjected to analysis of variance (ANOVA) using the Statistical Analysis System (SAS) university edition (SAS Institute Inc., Cary, NC). The differences between means were separated by Tukey's honestly significant difference (HSD) test at $p<0.05$. The results were expressed by means followed by the standard errors.

3. Results

Leaf morphology and anatomy response to growth temperature

At the end of the experiment, the significant differences in leaf dimensions ($p < 0.0001$) and dry matter ($p<0.0001$) between growth temperature levels were observed (Table 1). 'KU-PP2' peach trees that were forced at 25°C had the longest leaf length, followed by the 20°C and the 30°C treatments, while the leaf width of each treatment was comparable (p= 0.0864). The stomatal density increased by 7% with the increase in growth temperature from 20 to 25°C and reached its maximum value at 25°C. However, raising the growth temperature from 25 to 30°C significantly diminished stomatal density by 32%. In contrast, the leaf dry matter content slightly increased when the growth temperature increased. Compared with 20 and 25°C, the 30°C treatment increased leaf dry matter by a mean value of 5% FW.

The growth temperatures not only changed the leaf morphological characteristics but also affected leaf anatomical traits (Table 1). The higher temperature significantly decreased the thickness of leaves, palisade mesophyll, and spongy mesophyll ($p <$ 0.0001). The leaves that were forced at the highest growth temperature (30°C) were thinner than those

Table 1 - Leaf morphological and anatomical characteristics, percentage of leaf dry matter (DM), and stomatal density of the 'KU-PP2' peach cultivar at the end of the experiment. The peach trees were grown under three growing temperatures (20, 25, and 30°C)

Parameter	Growth temperature			*p-value*
	20°C	25°C	30°C	
Leaf length (cm)	17.1 ± 0.6 a [z]	18.6 ± 0.2 a	13.7 ± 0.5 b	< 0.0001
Leaf width (cm)	4.6 ± 0.1	4.5 ± 0.1	3.9 ± 0.3	0.0864
Percentage of leaf dry matter (% FW)	44.9 ± 1.35 b	44.5 ± 0.46 b	50.8 ± 0.56 a	< 0.0001
Leaf thickness (µm)	58 ± 1.6 a	41 ± 1.0 b	37 ± 1.1 b	< 0.0001
Adaxial epidermis thickness (µm)	6 ± 0.3	5 ± 0.3	5 ± 0.5	0.7828
Abaxial epidermis thickness (µm)	4 ± 0.3	4 ± 0.4	3 ± 0.2	0.4418
Palisade thickness (µm)	28 ± 0.7 a	18 ± 0.4 b	15 ± 0.2 c	< 0.0001
Spongy thickness (µm)	20 ± 0.5 a	13 ± 0.6 b	14 ± 0.6 b	< 0.0001
Stomatal density (no. mm^{-2})	242 ± 2 b	260 ± 3 a	176 ± 4 c	< 0.0001

[z] Data are mean values ± standard errors (n = 10). The different lowercase letters within the same row indicate significant differences at p ≤ 0.05 (Tukey's test).

from the trees grown at 25 and 20°C, as well as palisade and spongy mesophyll layers. On the other hand, the different growing temperatures did not significantly change the adaxial and abaxial epidermis thickness (p= 0.7828 and p= 0.4418, respectively) throughout the temperature treatments. Figure 1 shows the light microscopy pictures of leaf cross-sections for all temperature treatments, measured at the end of treatment.

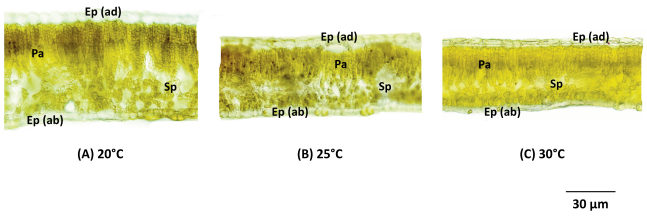

Fig. 1 - Anatomical comparison of leaf cross-section of 'KU-PP2' peach trees under (A) 20°C, (B) 25°C and (C) 30°C at the end of the experiment. The cross-sections of samples were observed and photographed under a light microscope. Ep (ad) = adaxial epidermis; Pa = palisade mesophyll layer; Sp = spongy mesophyll layer; and Ep (ab) = abaxial epidermis. These pictures were taken on 15 November 2019.

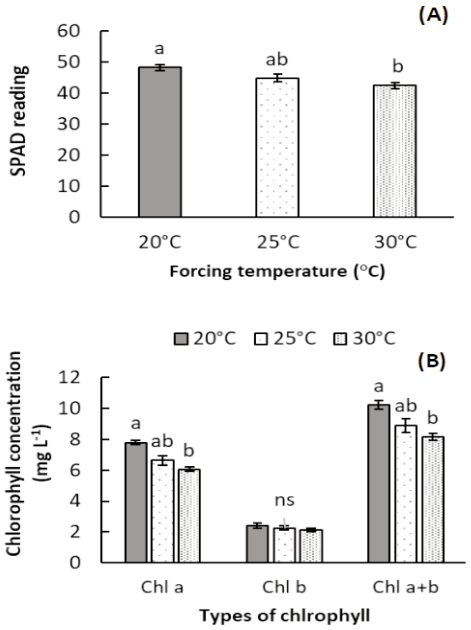

Fig. 2 - SPAD value (A) and chlorophyll content (B) in response to growth temperature treatments. Data represent means ± standard error (n= 5). Different letters indicate significant differences according to Tukey's test (p < 0.05) and NS denotes non-significant.

SPAD values, chlorophyll contents, photosynthetic rate, and gas exchange parameters

Figure 2 shows the high-temperature conditions caused a reduction in SPAD values (p= 0.0206) and loss of Chl content, especially Chl *a* (p= 0.0029) and Chl *a+b* (p= 0.0133). The SPAD reading for the 30°C treatment showed decreases by 12.5%, compared with the 20°C treatment. However, the SPAD values for the 20 and 30°C treatments were not significantly different from that of the 25°C treatment. Similarly, the Chl content decreased when exposed to an increasing temperature compared with the 20°C treatment. The maximum reduction in Chl *a* content (22.3%) occurred with 30°C, the Chl *a+b* concentration for 30°C decreased by 20.1%, while the Chl *b* content was not significantly affected (p= 0.3494).

The responses of the net photosynthetic rate (P_n) and the gas exchange parameters to growth temperature differed significantly depending on the levels and duration of the temperature treatments (Fig. 3). One week after temperature treatment started, the P_n for all treatments increased considerably

(p<0.0001). The P_n of the 30°C treatment was higher than the other treatments in this period (p=0.005). Subsequently, the P_n of the 20 and 25°C treatments steadily increased and remained stable at a higher level than at the beginning of treatment until the harvesting period. Conversely, the P_n of the 30°C treatment dramatically declined in the second week (p=0.0007) and after that gradually decreased and reached its lowest level in the eighth week (p<0.0001) after temperature treatment started (Fig. 3A). The average P_n values of the mature leaves under the 20°C treatment was higher than those in the 25 and 30°C treatments by 12.8 and 47.7%, respectively (p<0.0001). The changes in stomatal conductance (*gsw*) of each treatment were similar to those of the P_n values. The maximum *gsw* for 30°C was observed in the first week after the beginning of treatment while the peak *gsw* for 20 and 25°C occurred in the third week (Fig. 3B). The averages of *gsw* in both lower-temperature treatments were not different (p= 0.2578). The *gsw* of the higher-temperature treatment rapidly decreased in the second (p<0.0001) and eighth weeks (p= 0.02) after treatment started. Similarly, the peak of leaf internal CO_2 concentration (C_i) was observed one week after the beginning of treatment (Fig. 3C). A higher growth temperature had greater effects on C_i, with a considerable reduction in C_i occurring twice; in the second and eighth weeks after treatment started (p<0.0001). The lower growth temperatures (20 and 25°C) had comparative effects on the values of transpiration rate (E). The mature leaf E under the 20 and 25°C treatments declined more slowly than under the 30°C treatment, with the average E for the lower temperatures (20 and 25 °C) being higher than that for the high-temperature treatment by 30−35% (p = 0.004; Fig. 3D).

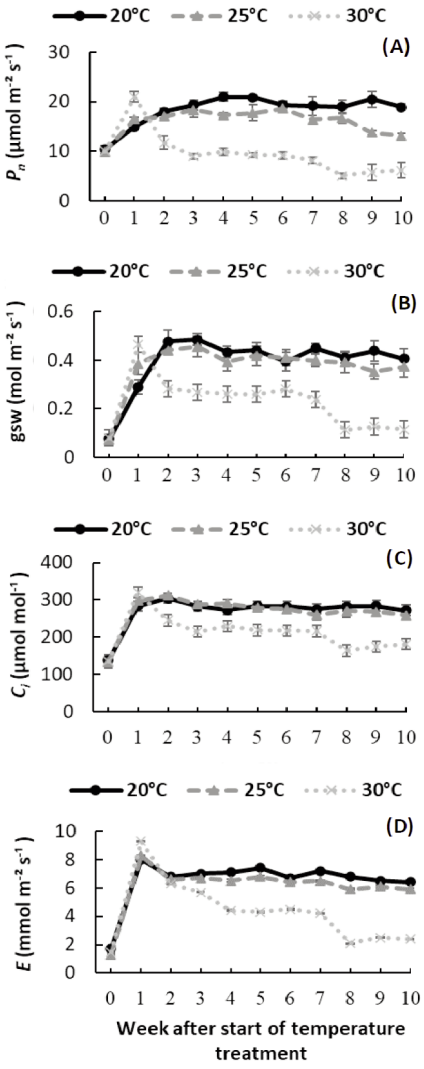

Fig. 3 - Effect of growth temperatures on (A) leaf net photosynthesis, (B) stomatal conductance, (C) leaf internal CO2 concentration, and (D) transpiration rate of the 'KU-PP2' peach trees. Data represent means ± standard error (n= 5).

Effect of growth temperature on fruit quality indexes

The morphological characteristics and chemical compositions of the ripe fruit are shown in Table 2. The results indicated that a high growth temperature strongly affected only the fruit morphological characteristics (p=<0.0001) of the 'KU-PP2' fruit and fruit weight (Table 2). However, significant differences in fruit shape (p = 0.0631) and chemical compositions of the fruit (p = 0.0881) were not found. An increase in growth temperature decreased fruit weight, fruit diameter, and fruit length. As shown in figure 4, there were significant contrasts in skin coloration for the 'KU-PP2' peaches with the different treatments. During the harvesting period, the fruit from the 30°C treatment showed a higher level of red coloration than the fruit from the 20 and 25°C treatments, indicating that increasing the temperature could accelerate the reddening of the fruit skin. Further, at 30°C, the fruit development period became shorter than under the 20 and 25°C conditions, with maturation occurring 14 days earlier.

4. Discussion and Conclusions

Long-term exposure to a moderate high-temperature regime (30°C) can result in cellular and physio-

Table 2 - Fruit quality characteristics of the 'KU-PP2' peach trees for each growth temperature treatment

Parameter	Growth temperature			p-value
	20°C	25°C	30°C	
Fruit weight (g)	164.34 ± 7.48 a [z]	131.89 ± 6.16 b	97.83 ± 7.05 c	< 0.0001
Fruit cheek diameter (mm)	68.1 ± 1.2 a	62.1 ± 1.4 b	56.2 ± 1.4 c	0.0002
Fruit suture diameter (mm)	69.9 ± 1.3 a	64.0 ± 1.0 b	58.5 ± 1.5 c	0.0002
Fruit length (mm)	59.9 ± 0.7 a	57.7 ± 1.0 a	50.0 ± 0.8 b	< 0.0001
Total soluble solids (°Brix)	15.0 ± 0.7	13.8 ± 0.2	13.3 ± 0.6	0.0881
Titratable acidity (g L^{-1})	0.21 ± 0.07	0.29 ± 0.01	0.24 ± 0.03	0.2331
Over color extent (%)	10–15 b	10–15 b	60–75 a	0.0013
Fruit development period (days)	96 a	91 a	81 b	0.0023

[z] Data are mean values ± standard errors (n = 10). The different lowercase letters within the same row indicate significant differences at p≤ 0.05 (Tukey's test).

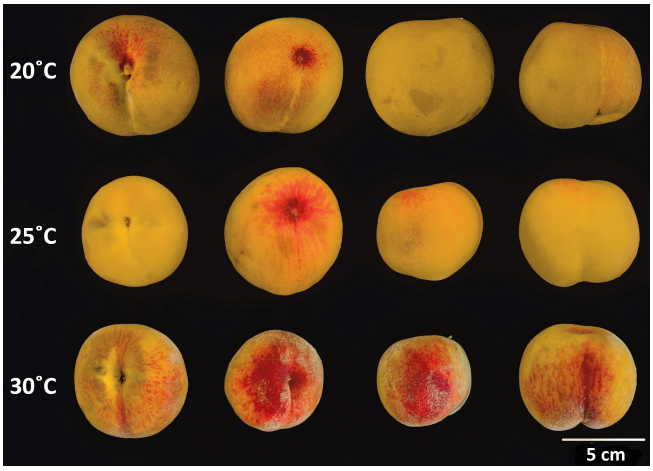

Fig. 4 - Effect of growth temperature on the coloration of the 'KU-PP2' fruits during the commercial ripening period.

logical adaptation of 'KU-PP2'. The responses of the peach trees to a high growing temperature could breakdown Chl, change leaf structure, reduce P_n, hasten fruit maturity, and could further explain the decrease in fruit quality under high temperatures in the present study. Similar to previous studies, high-temperature conditions induced closure of the stomata and generation of reactive oxygen species (ROS), damaged chloroplast structure and PS II and decreased photosynthetic pigments and enzyme activities (Takahashi and Murata, 2006; Ashraf and Harris, 2013; Chen *et al*., 2017; Jumrani *et al*., 2017).

Under high-temperature regimes, plants avoid heat damage and reduce excessive energy absorption on their leaves by decreasing leaf size, covering leaf surfaces with a thick waxy cuticle as well as trichome, changing leaf shape, or increasing the number of stomata. Small leaves can also reduce water loss and have less surface area exposed to solar radiation (Hasanuzzaman *et al*., 2013). Plants with thinner leaves and high stomatal densities can evacuate heat to the environment quicker than large leaves. A similar response to high temperatures was found in this study, in which leaf size and thickness of the leaf blades, including the epidermal and mesophyll layers, decreased. Elevating the temperature from 20 to 25°C increased the stomatal density, but the number of stomata sharply decreased when the growth temperature increased from 25 to 30°C. Previous study found similar results: the stomatal density of blueberry decreased when the temperature exceeded the optimum growth temperature (Hao *et al*., 2019). They suggested that increasing stomatal density may be an efficient strategy for evacuating more heat by evaporative cooling, but this strategy is inefficient under higher temperatures (Xu, 2015). It has been reported that a high temperature limits CO_2 and H_2O diffusion, resulting in increased resistance to gas exchange (Mukohata *et al*., 1971; Monson *et al*., 1982). In this study, we found that the transpiration rate (*E*) of the leaves under the 30°C treatment sharply decreased at four weeks after temperature treatment started, while the *E* of the leaves under both the 20 and 25°C treatments remained constant or slightly increased. The reduction in *E* under high-temperature conditions reflected the low efficiency of leaf cooling. In other words, the convective processes of heat through transpiration were reduced, resulting in excessive leaf temperature above an optimum point. The trees grown at 25°C tended to maintain transpiration cooling by increasing stomatal density, which reduces the negative

effects of excessive heat on their foliage, leading to the maintaining of high *E* and P_n.

The higher temperature decreased the concentration of Chl *a* and Chl *a+b*. As was also observed in this study, Chl contents have been reported to be sensitive to high-temperature conditions. The decline in Chl pigments may correlate to impaired Chl biosynthesis, exacerbated Chl breakdown, or both. The inhibition of Chl biosynthesis and the increase in Chl degradation under high temperature results from the destruction and construction of several enzymes (Efeoglu and Terzioglu, 2009). Additionally, the reduction in Chl content observed under high temperature is associated with physical damage to thylakoid membranes by excessive ROS accumulation (Halliwell and Gutteridge, 2007). Chl is embedded in the thylakoid membranes; therefore, damage to these membranes could result in Chl loss (Mathur *et al*., 2014; Chen *et al*., 2017; Jumrani *et al*., 2017). The imbalance between Chl biosynthesis and degradation disrupts the photosynthesis apparatus resulting in decreased photosynthetic efficiency, eventually influencing plant growth and fruit quality (Shanshan *et al*., 2020).

Changes in P_n have been directly linked to the level and duration of high-temperature exposure (Hao *et al*., 2019). In this study, one week after temperature treatment started, the P_n of 'KU-PP2' increased rapidly with the initial rise in growth temperature; as the forcing condition continued, P_n under a moderately high-temperature treatment (30°C) dramatically decreased, whereas the P_n under both the 20 and 25°C conditions steadily increased and remained constant until the harvesting period. The response of P_n to growth temperature can depend on two factors - non-stomatal and stomatal (Cui *et al*., 2006; Chen *et al*., 2014), which can be indicated by the difference in *gsw* and C_i patterns (Farquhar and Sharkey, 1982). If *gsw* decreased or stabilized but C_i increased, the decline in P_n can be attributed to non-stomatal factors. If both *gsw* and C_i decreased simultaneously, P_n could be ascribed to stomatal factors. In this study, the increase in P_n and C_i at the onset of treatment may result from the increase in enzyme activities in the photosynthetic system catalyzed by high temperatures. Therefore, an increase in P_n in this period could be identified as a non-stomatal factor.

Furthermore, the decrease in P_n under the prolonged higher-temperature treatment (30°C) can be divided into two periods: 2-7 weeks and 8-13 weeks after the onset of forcing. For 2-7 weeks, the decrease in P_n can be ascribed to a non-stomatal limitation, with *gsw* significantly decreasing and C_i increasing. The non-stomatal factors play a role in the reduction of P_n in the 2-7 weeks period after temperature treatment started through damage to the structures of the chloroplast, impairment of Chl biosynthesis, and increased Chl degradation. This hypothesis is supported by the reduction in Chl *a* and Chl *a+b* observed in this study. With exposure to forcing conditions over an extended period, P_n, C_i, and *gsw* of the 30°C treatment gradually decreased and reached their lowest levels in the eighth week after temperature treatment started, indicating that P_n in this period might be limited by stomatal factors through changes in stomatal density and modified leaf morphological and anatomical characteristics. Our study showed that the size and thickness of the leaves, including the epidermal and mesophyll layers, decreased with the elevated growth temperature, and thus led to the decline in P_n as stomatal limitations.

The differences in fruit morphological characteristics, such as fruit weight and fruit size of the trees under high-temperature conditions, might be associated with the decline in the fruit development period and P_n. Previous studies reported that the relationships between fruit development period (FDP) and fruit weight and diameter were observed in apple and peach (Sugiura *et al*., 2013; Giovannelli *et al*., 2014). In the 30°C treatment, fruit size was lower than those in the 20 and 25°C treatments, which was expected according to the length of their FDP. Additionally, previous studies indicated that a low P_n causes a steep reduction in fruit size because most of the energy used in fruit development is generated via photosynthesis during the year (Pavel and DeJong, 1993; Grossman and DeJong, 1995) Similarly, Lopez and DeJong (2007) reported that high temperature during fruit development increases the potential of fruit growth without enough resources to subsidize fruit growth, resulting in smaller fruit size. High temperatures not only depress photosynthesis but also increase leaf respiration. Plants grown under high-temperature conditions may consume much more energy because of increased leaf respiration caused by increased temperatures (Corelli-Grappadelli and Lakso, 2004; Hao *et al*., 2019). This result is supported by the increase in both the number and size of mitochondria in *Arabidopsis* thaliana, indicating that more starch and soluble sugar are consumed by leaf

respiration and rapid growth because of increased temperature (Jin *et al*., 2011). Hence, the reduction in fruit size of 'KU-PP2' grown under high temperatures may be supported by the above conclusion.

In this study, we found that the red coloration in 'KU-PP2' peel at 30°C was higher than those at 20 and 25 °C. Previous study showed the red coloration in plum (*P. salicina* Lindl.) peel increases under high-temperature conditions (35°C) (Junping *et al*., 2017). Conversely, the biosynthesis of anthocyanin in grape and apple is suppressed by high temperatures (Lin-Wang, 2011; Mori *et al*., 2017). Junping *et al*. (2017) showed that high temperatures can stimulate red skin coloration in plum by increasing respiration and ethylene production. Long-term forcing under high-temperature conditions may increase the respiration rate in 'KU-PP2' peach fruits, and hence enhance red coloration in the fruit peel. Moreover, the development of red coloration in peach fruit skin is positively related to light conditions (Corelli-Grappadelli and Coston, 1991; Kataoka and Beppu, 2004). Previous study showed that 'Redhaven' peach fruits that develop in the shade have less red coloration than those that develop in full sunlight (Erez and Flore, 1986). In our study, the fruit grown under the 30°C treatment had smaller leaves, leading to a decrease in canopy shade. Thus, the peach fruits grown under the 30°C treatment were exposed to more sunlight, which might result in higher red skin coloration.

In conclusion, this study illustrated the effect of growth temperature on plant development and fruit quality of 'KU-PP2' peach trees. Air temperatures directly affect leaf morphology, leaf anatomy, and the photosynthetic ability of plants. The decline in carbon assimilation due to exposure to excessive temperatures could diminish the plant's ability to efficiently support fruit development, resulting in low yield and poor fruit quality. All these data show high-temperature stress in the 'KU-PP2' peach cultivar caused by long-term exposure to moderately high temperatures. Therefore, a better understanding of plant adaptability to high temperatures is crucial for growing low-chill peach cultivars in plastic houses with a heating system.

Acknowledgements

This research was funded by Japan Society for the Promotion of Science (JSPS) KAKENHI, grant number 18K05621.

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Adv. Hort. Sci., 2021 35(3): 243-253 DOI: 10.36253/ahsc-8376

AHS
Advances in Horticultural Science

Genetic diversity assessment of ancient mulberry (*Morus* spp.) in Lebanon using morphological, chemical and molecular markers (SSR and ISSR)

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Citation:
KADRI A., SALEH S., ELBITAR A., CHEHADE A., 2021 - *Genetic diversity assessment of ancient mulberry (Morus spp.) in Lebanon using morphological, chemical and molecular markers (SSR and ISSR).* - Adv. Hort. Sci., 35(3): 243-253

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 24 March 2020
Accepted for publication 10 June 2021

Key words: Germplasm, microsatellite markers, morphological descriptors, polymorphism, principal component analysis.

Abstract: Lebanon has ancient mulberry trees which are the remnants of the abundant orchards that dominated its lands during the nineteenth century. Lebanese mulberry germplasm has not been assessed yet. This study aims to collect local old rainfed mulberry accessions from different geographical regions and assess their diversity by using morphological and molecular markers (SSR and ISSR). Genetic diversity of 70 accessions of mulberry were evaluated by using 27 morphological traits. The dendrogram based on the morphological attributes showed a relative separation of the different accessions based on fruits color and taste. Molecular analysis was performed for the accessions by using selected SSR and ISSR primers. The primers marked a high discriminating power (0.7 to 0.89). The dendrogram constructed on the base of UPGMA method showed 13 different groups. The clustering patterns indicated no location nor local name specificity among mulberry accessions. The combination of SSR and ISSR primers was informative for estimating the extent of mulberry genetic diversity. It can be concluded that there is a high level of genetic diversity within mulberry trees in Lebanon. These results will be useful for mulberry germplasm management in terms of biodiversity protection and as a valuable source of gene pool for crop improvement.

1. Introduction

Mulberry belongs to the genus *Morus* of the family Moraceae. It is a

multipurpose tree with a significant ecological, nutritional and economical high value. Mulberries are highly adaptable species in different soil and climatic conditions. They are generally quite tolerant to drought, pollution and poor soil. Therefore, they can be found in a wide area of tropical, subtropical, and temperate zones in Asia, Europe, North America, South America, and Africa (Kafkas *et al*., 2008). The genus *Morus* regroups 24 species (Thabti *et al*., 2014). The most widespread species in the Mediterranean climate areas are: *Morus alba* with fruit colors ranging from white to dark red, *Morus rubra* with mainly red/purple fruits and *Morus nigra* with dark purple to black fruits (Gerasopouls and Stravroulakis, 1997).

Mulberry fruits have remarkable potential for providing various valuable industrial products of high economic value for human beings. They are used for direct fruit consumption (*Morus alba*, *Morus indica*, *Morus nigra*, and *Morus laevigata*). Most of mulberry species have distinct flavor with juicy and acidic characteristics making them attractive for use in the processing industry for products such as fruit juice, ice cream, jelly, and jam (Ercisli and Orhan, 2007). Interest in mulberry has increased considerably over the last 20 years as a healthy fruit. *Morus* species have great antioxidant potential due to their high content in phenolic compounds including flavonoids, anthocyanins, and carotenoids (Zhang and Ma, 2018). Mulberries present anticancer and anti-inflammatory properties and show as well significant effect on many chronic diseases like diabetes (Nakamura *et al*., 2009; Kwon *et al*., 2015; Qian *et al*., 2015).

Mulberry is an economically important plant used for sericulture. It is the sole food plant for the domesticated silkworm, *Bombyx mori* (Zhao *et al*., 2009). The genus *Morus*, is cultivated extensively in East, Central and South Asia for silk production (Awasthi *et al*., 2004). Hence, mulberry is one of the most important components that decide the sustainability of this industry (Liu *et al*., 2009).

At the turn of the century, Lebanon was known for its high-quality silk industry. Bestowed with an ideal climate and a fertile soil, mulberries were planted everywhere in Lebanon and mulberry production flourished (Firro, 2009). The silk tradition in Lebanon is more than two thousand years old. It goes back to the period of the famous purple dye (Ourjouan) extracted from the Murex shell by the Phoenicians of Sidon and Tyre and used to produce imperial purple silk (Khater, 2009). In the 19th century, silk industry constituted almost 80% of Lebanon's economy. By the early 20th century, 70% to 80% of the cultivable land of the country mountainous regions (Mount Lebanon) became devoted to mulberry orchards. Due to the high demands in silk production, mulberry tree has an unsurpassed economic impact on rural communities. After 1940's, when silk began to be imported from the Far East, the sericulture industry declined sharply. Mulberry cultivation became marginalized. However, Lebanon still has very old rainfed mulberry trees which are the remnants of the abundant orchards that were once shaping the landscape of many villages. Mulberry trees are found in different Lebanese villages, mostly located at orchards periphery or in small gardens.

In Lebanon, mulberry genotypes are very diverse, as they were sometimes obtained in the past from seeds or from cuttings. This process has led to a great number of landraces adapted to different conditions and different uses throughout the country. In Lebanon, there are many local traditional accessions but no named cultivars. Mulberries are distinguished and denominated according to the fruit color: "Abyad" (white mulberry), "Mwachah" (purple mulberry), "Shami" and "Aswad" (black mulberries).

Mulberry genetic diversity is progressively being lost in farmers' fields and in nature. The threat results from the interaction of several factors and is processing at an alarming rate. The most crucial factors are urbanization, climatic changes, out breaks of new diseases and pests, and the frequent occurrence of natural calamities. Little information is available about the genetic diversity of Lebanese mulberries. To protect mulberry in Lebanon, a marginalized species, conservation programs should be initiated. In this study, we have collected local mulberry accessions from different geographical regions of Lebanon and assessed their genetic diversity by using agro-morphological traits as well as molecular markers (SSR and ISSR).

2. Materials and Methods

Field survey

Samples of fruits for morphological and chemical analyses were collected from local trees of mulberry *Morus* from 21 sites covering different Lebanese regions (the North plain, Bekaa plain, Mount

Lebanon, the South). These sites are subjected to different climatic conditions (precipitation, temperature) and agricultural practices. They are situated at an altitude between 30 and 1620 m, a latitude ranging from N33° 16' 166" to N34° 21 '51.5" and a longitude between E36°10' 849" and E35° 01' 38.7" (Fig. 1). The number of individual trees sampled per site (population) ranged from two to sixteen cultivars. In total, 70 accessions of mulberry were studied. Collected samples consisted of mature fruits (approximately 500 g) and vegetative materials (young leaves, mature leaves and branches). The studied accessions included 'Abyad' (white mulberry), 'Mwachah' (purple mulberry), 'Shami' and 'Aswad' (black mulberries).

Fig. 1 - Geographic distribution of the studied mulberry accessions as visualized with DIVA-GIS program (Hijmans *et al*., 2001).

Morphological and chemical characterizations

The characterization of the vegetative materials and the fruits was based on descriptors for investigation of mulberry germplasm's morphology produced by Agriculture and Consumer Protection FAO (Sohn, 2003). Thus 27 morphological characters were studied for the mulberry accessions. These studied traits included 13 qualitative characters (for the leaf: shape, margin, base, apex, surface, color, glossiness, phytotaxis, bud shape and color; for the fruit parts: shape, color, taste, seed color) and 14 quantitative characters (for the vegetative parts: leaf length, width and thickness, petiole length, bud length, internode length; for the fruit parts: fruit length, diameter and weight, peduncle length, juice percentage (volume of the juice*100/weight of the fruits), sugar quantity (using refractometer), pH and acidity (by titration reaction).

Molecular characterization

DNA extraction. Genomic DNA was extracted from mulberry young fresh leaves using cetyl trimethyl ammonium bromide (CTAB) procedure described by FAO/IAEA (2007). The DNA quantity and quality was visually quantified using the agarose gel electrophoresis method as described by Maniatis *et al*. (1982). DNA samples were stored at -20°C.

PCR amplification of the DNA with ISSR primers. Six primers (UBC807, UBC810, UBC826, UBC827, UBC864 and BI3) were tested for DNA amplification (Emir, 2013). The ISSR (Inter Simple Sequence Repeat) amplification was carried out as per Vijayan and Chatterjee (2003) using 20 µl reaction mixture containing 2 µl of 10 X PCR buffer (750 mM Tris-HCl pH 8.8; 0.1% Tween-20), 0.2 mM dNTP, 2 Mm $MgCl_2$, 200 nM Primer, 50 ng genomic DNA and 1 U Taq DNA polymerase (MBI Fermentas Inc, Hanover, MD-21076, USA). The PCR schedule included an initial cycle at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min and a final extension of 10 min at 72°C. The PCR products were resolved by electrophoresis on a 1.5% agarose gel in 1 X Tris Boric Acid buffer (TBE), stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

PCR amplification of the DNA with SSR primers. Microsatellite polymorphisms were identified using three SSR primers (primers: MulSTR1, MulSTR2 and MulSTR3) (Tikader *et al*., 2009). Microsatellite amplification reactions were performed in a final volume of 25 µL in the presence of 2.5 µl of buffer, 200 µM of each dNTP, 0.4 µM of each primer pair, 1 unit (U) of Taq DNA polymerase, 50 ng template DNA, and 2 mM $MgCl_2$. The amplification reaction consisted of an initial denaturation step at 94°C for 4 min, followed by 45 cycles of 1 min denaturation at 94°C, 65 sec annealing at 50°C, 90 sec extension at 72°C with a final extension of 72°C for 10 min using thermal cycles. The PCR amplification products were separated on a 6% denatured polyacrylamide gel and visualized by silver staining.

Data analysis

For qualitative traits, scores were attributed

according to FAO mulberry descriptors. A phenotypic diversity index, hsj (Shanon index) (Magurran, 1988) was calculated for each site to describe the phenotypic diversity of mulberry. The following formula was used for calculating hsj for each trait with n categories hsj=Σ $P_i LnP_i$ where p_i is the relative frequency in the *i*th category for the *j*th trait. The average diversity (H) over k traits of each site was estimated as: H=Σ hsj/k. Traits evaluation was performed by using the Principal Component Analysis (PCA). The relationships between mulberry leaves and fruits based on their quantitative and qualitative traits were studied using Hierarchical Cluster Analyses executed using Euclidean Distance following the Ward's method implemented in PAST software (Hammer *et al*., 2001).

To assess the information given by SSR and ISSR markers, the following parameters were calculated: number of alleles per locus, percentage of observed heterozygosity (Ho), expected heterozygosity (He= 1-Σpi^2, where pi is the frequency of the i^{th} allele) and the power of discrimination (PD = 1 -Σgi^2, where gi is the frequency of the i^{th} genotype). Genetic distances were calculated according to Jaccard (1908). Trees were produced by clustering the data with the unweighted pair-group method (UPGMA) with SAHN-clustering and tree programs of PAST software (Saporta, 1990).

3. Results

Mulberry trees were distributed over various agro-climatic areas of Lebanon (Fig. 1, Table 1). A total of 70 mulberry accessions were studied belonging to 'Abyad' (22 accessions), 'Mwachah' (25 accessions), 'Shami' (20 accessions) and 'Aswad' (3 accessions). Among mulberry species found in Lebanon, *Morus alba* was the dominant species in cultivation (95%). Around 85% of the surveyed mulberry trees were rainfed, old and inherited from family.

Morphological analysis

Leaves morphological characterization. Mulberry trees tend to have short trunks with large, low, spreading limbs. Leaves were alternately arranged and simple. The majority of cultivars had a cordate leaf shape, except the leaves of two accessions were reniform ('Abyad' and 'Shami') and two other were cordate to oval ('Abyad' and 'Mwashah'). All leaves

Table 1 - Climatic and geographic characteristics of the 21 locations surveyed to characterize the Lebanese mulberry trees

Site	Latitude (N)	Longitude (E)	Altitude (m)	Annual average temperature (°C)	Annual Rainfalls (mm)	Varieties	Number of accessions
Douris	33°59'588"	36°10'849"	1131	14.9	441	Abyad Mwachah	4
Rayak	33°51'751"	35°59'591"	927	15.1	544	Aswad	2
Nabishet	33°52'10.4"	36°06'34.7"	1233	13.6	570	Abyad Mwachah Sami	6
Jenta	33°51'23.5"	36°06'26.2"	1114	13.2	580	Abyad Mwachah	2
Britel	33°56'02"	36°08'54.3"	1154	14.7	471	Abyad Mwachah Shami	4
El borjein	33°39'27.2"	35°29'11.3"	1620	12.7	630	Shami	1
Baassir	33°39'30.1"	35°26'54.7"	1094	12.9	630	Abyad Mwachah Shami	2
Hawsh Nabi	33°55'28.6"	36°04'23.8"	990	15.2	544	Abyad Mwachah	2
Hawsh Refaa	33°55'23.7"	36°02'34.2"	971	15.1	530	Abyad Mwachah	5
Kfar Dabash	33°56'43.2"	36°02'13.7"	1079	15.1	540	Abyad Mwachah	1
Chmistar	33°57'49.6"	36°01'07"	1145	14.9	550	Shami	2
Beit Chama	33°55'07"	36°01'25"	1011	15.2	541	Abyad Mwachah	1
Tamnen taata	33°52'43.3"	35°59'45.9"	937	15.1	542	Abyad Mwachah	1
Chlifa	34°05'109"	36°06'098"	1012	14.7	461	Abyad Mwachah Shami	5
Flaoue	34°04'934"	36°03'761"	1139	14.6	461	Abyad Mwachah Shami	3
Dayr Lahmar	34°07'077"	36°07'940"	1012	14.5	461	Baladi	1
Zahle	33°48'59.9"	35°57'32.6"	882	15.2	646	Abyad Mwachah Shami	5
Ali ennahry	33°51'21.04"	35°01'38.7"	958	15.1	544	Shami	1
Sour	33°16'166"	35°13'133"	30	20.2	697	Abyad Mwachah Shami	4
Kfar Chakhna	34°21'51.5"	35°51'50.7"	198	13.9	754	Shami	2
Hasbaiya	33°32'74"	35°64'373"	467	15.1	590	Abyad Mwachah Shami	16

had dentate margins. They presented mainly a cordate base and an acute apex. Leaf surface of 70% of the accessions was slightly rough. Only nine accessions of 'Shami' presented rough surfaces.

Leaves generally presented an average length between 6.83 and 18.9 cm and width between 3.94 and 17.02 cm. Petiole length average was between 0.72 and 5.07 cm. Leaves of black mulberry accessions 'Shami' (0.02 and 0.03 cm) were thicker than those of white mulberries (0.01 cm).

Fruits morphological characterization. For the 70 accessions, the pomological characteristics investigated showed a great diversity. Concerning the fruit shape, 35.7% of the mulberry fruits had oblong shape, 27.1% were round, 24.2% were reniform, and 13% were oval. For the local variety 'Mwashah', nearly half of the accessions had oblong shape while the majority of 'Shami' had round one. White mulberry 'Abyad' presented mainly oblong and reniform shape.

The accessions showed significant differences in the fruit weight ranging from 1.1 g ('Abyad' from Doris) to 7.9 g ('Mwashah' from Hawshrefaa). Fruit length varied from 1.7 ('Shami' from Flewa) to 4.9 cm ('Mwashah' from Hawshrefaa-Bekaa) and fruit width from 1.1 ('Abyad' from Doris) to 2 cm ('Shami' from Baaser). Minimum length of fruit peduncle was 0.11 ('Shami' from Flewa) and maximum length 1.28 cm ('Aswad' from Tyr).

Fruit color of mulberry accessions was diverse: 'Abyad' accessions were white and 'Mwachah' accessions were violet. The fruit color of 'Shami' accessions were darker and varied between red-purple to black or black. Seed color varied between light yellow and yellow-brown; 'Mwashah' fruits had mainly light yellow seeds and 'Shami' presented yellow-brown seeds.

The percentage of juice yields differed within the accessions of the same local variety. The lowest and greatest juice yields varied from 30.1% ('Abyad' from Nabishit) to 72.3% ('Shami' from Baaser) and 73.1% ('Abyad' from Shlifa). As for the chemical characteristics of mulberry accessions, sugar content ranged from 7 ('Mwashah' from Janta) to 19.5 Brix ('Aswad' from Tal Amara). pH varied widely from 2.29 ('Abyad' from Hasbaya) to 6.42 ('Abyad' from Douris). Titrable acidity was very diverse in the different mulberry accessions. Titrable acidity values were from 0.01 ('Mwashah' from Zahle) to 0.14 g/l ('Shami' from Flewa).

Morphological characterization PCA

The characterization of the collected mulberry accessions using different morphological characters showed high level of variation among the accessions. The Principal Component Analysis (PCA) revealed that the first 3 components explained 37% of the total variation, based on the 27 morphological characters (Table 2). The first component represented 18 % of the total variation and included fruit and leaves characteristics. It comprised fruit length, color, taste, pH, acidity and peduncle length, besides to the petiole length, glossiness and thickness of leaves. The second component represented 10% of the total variation and is mainly influenced by leaf width. The third component was characterized by a percentage of variation of 9% and is dominated by the bud length character.

Table 2 - Principal component analysis (PCA) of the 27 morphological characters evaluated for the 70 different mulberry accessions. The characters in bold are discriminant

Variables	Factor 1	Factor 2	Factor 3
Fruit length	**-0.622011**	-0.428543	0.385704
Fruit weight	-0.422828	-0.548276	0.534493
Fruit diameter	-0.279504	-0.469198	0.362977
Peduncle Length	**-0.639179**	-0.10646	-0.393485
Percentage of	0.126663	0.055644	0.121337
sugar quantity	0.050968	-0.124287	-0.168429
pH	**-0.720232**	0.290375	0.027219
Acidity	**0.605034**	-0.131547	0.165099
Leaf length	-0.014178	-0.543281	-0.48354
Leaf width	-0.038798	**-0.719527**	-0.23667
Petiole length	**-0.700726**	-0.161604	-0.190875
Leaf thickness	**-0.739758**	-0.294618	0.241539
Date of maturity	-0.085013	-0.253214	0.241209
Bud length	0.069769	-0.177441	**-0.629778**
Bud width	0.136856	-0.265182	-0.59383
Bud shape	-0.192576	0.061054	-0.048574
Internodal distan-	-0.141876	-0.525259	-0.327898
Leaf shape	-0.109165	-0.008143	-0.223988
Leaf base	-0.074223	0.208101	0.302694
Leaf apex	0.007149	-0.251893	-0.077977
Leaf surface	-0.50104	0.446809	-0.164365
Leaf color	-0.260375	-0.135751	0.031335
Leaf glossiness	**0.670155**	0.023403	-0.053846
Fruit shape	-0.498979	-0.040292	0.105085
Fruit color	**-0.6271**	0.204057	-0.072799
Fruit taste	**0.663459**	0.058647	0.089481
Seed color	-0.210256	-0.238095	0.409438
Exp.Var	4.797.858	2.719.786	247.986
Prp.Tot	0.177698	0.100733	0.091847

Classification of accessions based on morphological attributes

The accessions could be separated into groups based on the 11 most discriminant traits. The hierarchical cluster analysis classified mulberry accessions in 6 groups at -6 similarity of Euclidean distance (Fig. 2, Table 3).

'Shami' accessions were classified separately into 3 main groups (G1, G4 and G5). The first group G1 included 'Shami' and 2 accessions of 'Aswad'. G1, G4 and G5 accessions were characterized by a sour fruit taste and a dark black-purple or black-red fruit color. Fruits of G4 and G5 presented significantly the lowest pH mean values (3.78 and 3.85 respectively). G5 fruits presented the lowest sugar content (9.62 °Brix). Accessions of G4 and G5 had the shortest fruit length (2.32 cm and 2.45 cm respectively) and the shortest peduncle (0.24 cm and 0.46 cm respectively). Regarding leaf characteristics, G4 and G5 accessions were characterized by low mean value of leaf length, while G1 were characterized by a significantly high leaf length (17 cm). G1, G4 and G5 accessions had also the thickest limb (0.02 - 0.03cm) and the shortest peduncle (1.4 - 1.48 cm).

The group G2 consisted of 12 accessions of 'Abyad' and one of 'Mwashah'. These accessions were characterized by a white fruit color and a sweet taste. The group G3 consisted of 19 accessions of 'Mwashah' and 2 of 'Shami'. They were characterized by purple fruits. They presented the highest value of fruit length (3 cm) and medium values for pH and

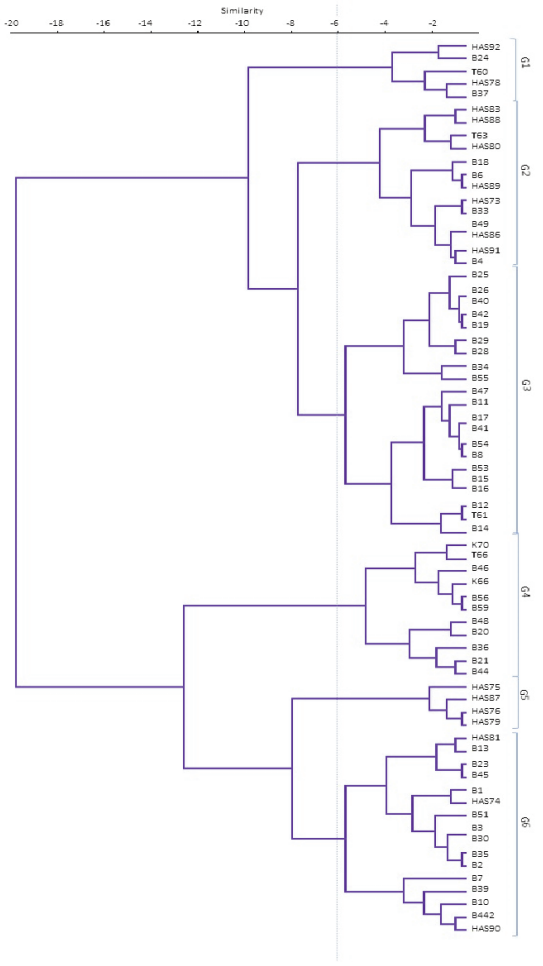

Fig. 2 - Dendrogram of Jaccard distance based on the characters that presented high variability in the principle component analysis, depicting the genetic relationship among the 70 different mulberry accessions.

Table 3 - Variability of the quantitative morphological fruit characteristics for the accessions clustered within the same group (G1, G2, G3, G4, G5 and G6) minimum, maximum and mean values (with standard deviation)

Group (number of accessions)	Fruit length (cm)	Fruit width (cm)	Fruit weight (cm)	Peduncle (cm)	Percentage of juice (%)	Sugar quantity (Brix)	pH	Acidity (g/l)
	2.11< L<4.05	0.96< w <2.05	0.99< W <7.81	0.93< P <1.28	51.4< J <72.3	7< S<15	3.4<pH <5.97	0.028< N < 0.13
G1 (3 Shami, 2Aswad)	2.91±0.81 a	1.59±0.42 a	3.91±2.60 a	1.13±0.14 a	59±7.77 a	11.6±3.84 bc	4.79±1.03 b	0.05±0.04 b
	2.14< L< 3.45	1.49< w <1.88	2.05< W <5.72	0.43< P <1.4	34.2< J <58.2	11< S<16.5	2.29<pH <6.47	0.018< N < 0.076
G2 (12 Abyad, 1 Mwashah)	2.77±0.39 ab	1.61±0.20 a	4.00±1.17 a	0.81±0.26 b	52.68±8.50 a	13.15±1.87 ab	5.70±1.28 ab	0.05±0.02 b
	2.22< L <4.95	1.4< w <2	2.6< W <7.98	0.51< P <0.99	35.4< J <64.1	7< S<15	3.5<pH <6.35	0.014< N < 0.116
G3 (19Mwashah, 2 Shami)	3.00±0.63 a	1.75±0.15 a	4.90±1.42 a	0.76±0.18 b	51.67±8.18 a	11.33±2.21 bc	5.47±1.04 ab	0.03±0.02 b
	1.71< L <3.1	1.3< w <1.86	1.3< W <5.38	0.1< P <0.90	36.2< J <76.7	7< S<18.5	3.14<pH <5.97	0.04< N < 0.2
G4 (11 Shami)	2.32±0.39 b	1.57±0.19 a	3.74±1.30 a	0.24±0.23 d	53.69±12.20 a	13.73±3.08 ab	3.78±0.39 c	0.10±0.04 a
	2.13< L <2.68	1.5< w < 1.93	3.4< W <5.77	0.38< P <0.56	49.4< J <58.5	8.5< S<11	3.1<pH <6.12	0.038< N < 0.05
G5 (4 Shami)	2.45±0.255 ab	1.72±0.19 a	4.68±1.09 a	0.46±0.09 c	52.95±4.09 a	9.62±1.11 c	3.85±1.51 c	0.045±0.006 b
	1.88< L <3.33	1.10< w <1.95	1.11< W <5.64	0.46< P <1.14	30.1< J <73.1	10.5< S<21	5.3<pH <6.51	0.014< N < 0.08
G6 (5 Mwashah 10Abyad, 1Aswad)	2.68±0.44 ab	1.59±0.25 a	3.76±1.26 a	0.82±0.22 b	50.74±10.31 a	14.91±3.07 a	6.05±0.45 a	0.041±0.019 b

Different letters were significantly different at the 0.05 level (Duncan's Multiple Range Test).

sugar content. The group 6 regrouped 5 accessions of 'Mwashah', 10 of 'Abyad' and one of 'Aswad'. These accessions were characterized by their sweet taste (high sugar content and high pH). Groups 2, 3 and 6 presented a medium leaf thickness.

Molecular characterization

ISSR analysis. The molecular analysis of mulberry accessions presented a high variability. The ISSR markers showed distinct polymorphism between the different mulberry accessions, only primers UBC-826 and UBC-864 showed no amplification. A total of 18 polymorphic bands were detected across the 70 accessions of mulberry through the use of four ISSR primers UBC807, UBC810, UBC827 and BI3. The size of amplified products ranged from 700 bp to 1600 bp. The number of scorable markers produced per primer ranged between 4 (UBC810, UBC827) and 5 (UBC807, BI3). This study showed that (AC), (GA) repeat primers generated excellent band profiles. Primers synthesized from (ATG) repeats failed to amplify.

The power of discrimination calculated for each primer (Table 4) enabled us to evaluate the genetic diversity of our locus. The calculated power of discrimination PD values were between 0.75 and 0.89, showing that the studied loci are of high diversity. These primers could be effectively used to study polymorphism between mulberry accessions.

SSR analysis. Primer MulSTR1 showed no amplification within our accessions. A total of 10 polymorphic bands were detected across the 70 accessions of mulberry through the use of two SSR primers (MulSTR2, MulSTR3). Upon using MulSTR2 SSR primer, the acrylamide gel showed three bands across the different accessions (192bp, 200bp, and 208bp), while MulSTR3 SSR primer generated 7 different bands ranging from 192bp to 275bp (Table 5). The results presented high polymorphism in all the amplified loci. In their assessment of mulberry genotypes by SSR marker profile, Wani *et al*. (2013) used MulSTR2 and MulSTR3 primers which generated each 2 alleles among the 17 mulberry genotypes tested. Our results showed a higher number of polymorphic bands for a higher number of accessions. The power of discrimination was relatively high for each primer, PD>0.5. MulSTR3 presented higher polymorphism than MulSTR2, with a PD of 0.8 and an expected heterozygosity of 0.713. These two primers and especially MulSTR3 could be effectively used in genetic diversity studies of mulberry.

Classification of accessions based on molecular markers

The allelic diversity data was used to produce a

Table 4 - Primer sequences, number and sizes (bp) of the produced bands and discriminating power (Dp) of the six ISSR markers used in the study

Primers	Sequence	Number of bands	Band sizes (bp)	Dp
UBC-810	3´GAGAGAGAGAGAGAGAT 5´	4	800-1500	0.80
UBC-807	3´AGAGAGAGAGAGAGAGT 5´	5	900-1600	0.89
UBC-827	3´ACACACACACACACACAG 5´	4	700-1700	0.85
BI3	3´ACACACACACACT 5´	5	500-1500	0.75
UBC-826	3´ACACACACACACACAC5´	-	-	-
UBC-864	3´ ATGATGATGATGATGATG5´	-	-	-

Table 5 - Primer sequences, number and sizes (bp) of the produced bands and discrimination power (Dp) of the three microsatellite markers used in the study

Primers	Sequence	Number of bands	Band sizes (bp)	Dp
MulSTR1	F: 5'GCCGTGTACCAGTGGAGTTTGCA 3' R:5'TGACCGTTTCTTCCACTTTACC-	-	-	-
MulSTR2	F:5´ CGTGGGGCTTAGGCTGAGTAGAGG R:5´ CACCACCACTACTTCTCTTCTTCCAG	3	192-208	0.52
MulSTR3	F: 5´ GGGTTGGGTAGATGGGCTTATGT- R:5´ CCCTATTAACTTTTTGGTCACCTCTA	7	192-275	0.83

dendrogram *via* the distance matrix-UPGMA (Fig. 3), thus revealing the genetic relationship among mulberry accessions. The dendrogram constructed on the base of the SSR and ISSR amplification product of the different mulberry accessions showed 14 different groups at the Jaccard distance of similarity 0.34. Clusters G1, G2, G3, G5, G6 and G10 regrouped accessions of the three varieties 'Mwashah', 'Shami' and 'Abyad'. G4 contained the three varieties as the previous groups in addition to an accession of 'Aswad'. One single accession constructed individually the groups G7 ('Shami'), G8 ('Abyad'), G9 ('Aswad'), G11 ('Shami') G12 ('Abyad'), G13 ('Mwashah') and G14 ('Abyad').

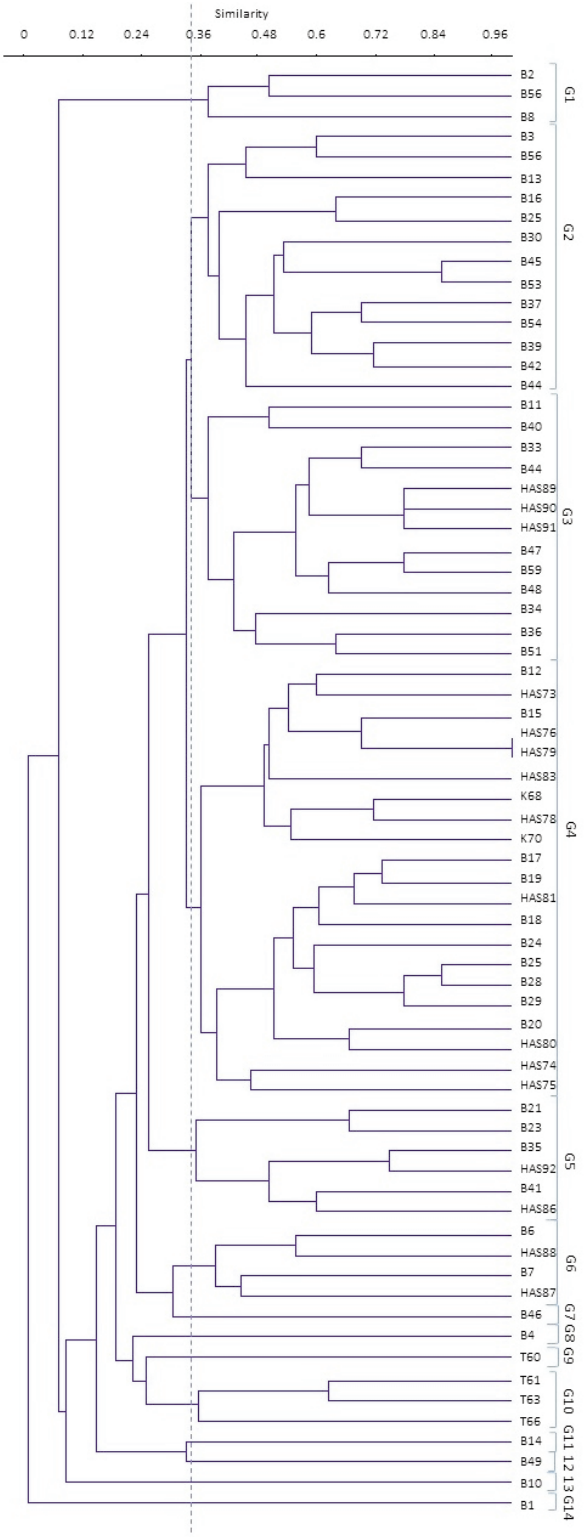

Fig. 3 - Dendrogram constructed from SSR and ISSR markers, using PAST program, Jaccard distance and UPGMA clustering of 70 mulberry accessions.

4. Discussion and Conclusions

The conservation of the genetic variability of the ancient Lebanese mulberry trees is of utmost importance for germplasm preservation and for future breeding programs. Lebanese mulberries germplasm has not been assessed yet. This study is the first genetic diversity assessment of the Lebanese mulberry germplasm using a set of morphological traits and genetic markers. Our inventories recensed accessions of four vernacular names 'Abyad', 'Mwachah', 'Shami' and 'Aswad' across different Lebanese regions. This shows that a limited number of traditional varieties was cultivated since decades, however morphological and molecular characterization of these 70 accessions revealed high diversity of this germplasm collection.

The results of morphological characterization revealed a high level of variation among mulberry characters. Among the 27 descriptors studied, 11 specific characters of fruits (Fruit taste, fruit length, fruit color, pH, titrable acidity and peduncle length) and leaves (petiole length, leaf thickness, leaf glossiness, leaf width and bud length) revealed to be the most discriminating characters. The first component of the PCA was dominated by the fruit characteristics. A broad morphological diversity of the fruit was reported for mulberry germplasm (Yilmaz *et al*., 2012; Peris *et al*., 2014; Aljane and Sdiri, 2016; Krishna et *al*., 2020). In our study, fruits exhibited distinct variations. Fruits shapes were diverse. Fruit color, titrable acidity, sugar content, juice yield and pH content were the most discriminating characters to differentiate mulberry accessions. Similar results were reported and significant differences were observed between the fruit characteristics (Yilmaz *et al*., 2012; Peris *et al*., 2014; Aljane and Sdiri, 2016; Krishna et *al*., 2020). Fruit color is a desirable character for commercial acceptance of a variety. Fruit

color of our accessions varied from white, red, purple to black. The percentage of juice yields were within the limits of Yilmaz *et al*. (2012) study (between 39% and 72%). All black mulberries had the highest fruit juice yield ratio, the highest acidity values and the lowest sugar content. This is consistent with previous researches (Ozdemir and Topuz, 1998; Gunes and Cekic, 2004; Aljane and Sdiri, 2016). Therefore, black mulberries are preferred for processing into juice. Black colored mulberry species received recently a great importance due to higher contents of phenolic compounds and to their delicious taste (Aljane and Sdiri, 2016).

The dendrogram constructed on the base of the most discriminant morphological characters divided the accessions into 6 distinct groups. The evaluation of the relationship among accessions reduced their differentiation to fruit color and taste. A non-negligible variability of other traits influenced the grouping involving the length of the fruit, leaf and peduncle. The accessions grouping was marginally correlated to the accessions vernacular names with many exceptions. The dendrogram revealed that accessions within each cluster belonged to different regions suggesting that there was no clear relationship between accessions and geographical diversity. This is the case of the group G4 that included 'Shami' accessions growing in North Lebanon (Kfarchakhna) and in South Lebanon (Sour). Such results have been reported in different crops by several studies, e.g. on chestnut (Marinoni *et al*., 2013), almond (Chalak *et al*., 2007; Halasz *et al.*, 2019) and olives (Chehade *et al*., 2015). This variability could be attributed to the free exchange of planting material between different Lebanese villages and emphasizes the adaptability of mulberry to different ecological conditions.

In this study, we evaluated the genetic diversity and the relationships among the collected mulberry accessions using SSR and ISSR markers. The results showed high polymorphism in all the amplified loci. The power of discrimination values was high showing that the studied loci are of high diversity. The observed SSR markers heterozygosity were high. Earlier studies using amplified fragment length polymorphism (Sharma *et al*., 2000), ISSR (Awasthi *et al*., 2004), and RAPD (Xiang *et al*., 1995; Feng *et al*., 1996; Zhao and Pan, 2000; Esha and Shirish, 2001) also showed a large genetic variation among different mulberry genotypes. Such a high level of polymorphism reflects the outcrossing nature of the species. In this work, the ISSR profiles generated by (AC) and (GA) repeat anchored primers showed that these repeats are abundant in our accessions. Vijayan and Chatterjee (2003) observed amplification of (AC) rich repeat based ISSR primers. Awasthi *et al*. (2004) concluded that (CA)/(TG) repeats are abundant in *Morus* genome.

Cluster analysis of SSR and ISSR data using UPGMA revealed high genetic distances between the studied accessions. Five groups were constructed by one single accession. The other clusters regrouped accessions of 'Mwashah', 'Shami' and 'Abyad' within each group. The distanced genetic relationships among mulberry accessions are in consistence with their high heterozygosity due to their outbreeding reproductive system (Dandin, 1998). Accessions from different varieties and from different sites were grouped together. The molecular results emphasized that genetic diversity among mulberry accessions is not influenced by their geographical origin nor by their local names. This finding is in agreement with other researchers who studied genetic diversity using SSR markers on different crops, almonds (Distefano *et al*., 2013), mung bean (Wang *et al*., 2018) and torch Ginger (Ismail *et al*., 2019). The analysis of the genetic parameters showed the high diversity of mulberry in Lebanon.

The comparison between morphological and molecular diversity indicated that morphological descriptors provide different information than the molecular one. In comparison with other works in woody species there were also no correlation. For example, two 'Shami' accessions (code B24 and HAS92) were in the same group in the morphological dendrogram however they belong to different groups in the molecular one. One 'Aswad' (black mulberry, code B6) accession and another 'Abyad' (white mulberry, code B7) accession were in the same group in the molecular dendrogram but they were not in the morphological one. It is probably that our markers sampled mainly a non-adaptive diversity.

The results of this study revealed a large morphological diversity and a high genetic variation among the Lebanese mulberry accessions. The combination of SSR and ISSR primers was informative for estimating the extent of mulberry genetic diversity. Morphological and molecular clusters have distinguished different lines of mulberry which may help in the selection of the most diverse profile. This germplasm would enhance the local gene pool and expand genetic variation for mulberry breeding program in the future.

Acknowledgements

The authors would like to thank Dr. Michel Afram for his support.

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Adv. Hort. Sci., 2021 35(3): 255-267 DOI: 10.36253/ahsc-9689

AHS
Advances in Horticultural Science

Genetic diversity in *Colocasia esculenta* and *Xanthosoma mafaffa* in Togo, West Africa

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Citation:
BAMMITE D., MATTHEWS P.J., DAGNON D.Y., AGBOGAN A., AGRE P., AKINTAYO T.O., ODAH K., DANSI A., ABBERTON M., TOZO K., 2021 - *Genetic and morphological diversity in Colocasia esculenta and Xanthosoma maffafa in Togo, West Africa.* - Adv. Hort. Sci., 35(3): 255-267

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 9 September 2020
Accepted for publication 9 July 2021

Key words: crop diversity, new cocoyam, SSR, taro, Togo.

Abstract: Taro and new cocoyam are root and leaf crops commonly grown in tropical to warm temperate regions. In Togo, they are neglected and underutilized. Here we report the genetic diversity of 26 accessions of taro and 101 accessions of new cocoyam. Analysis of simple sequence repeats revealed low polymorphic information content of 0.43 and 0.25 in taro and new cocoyam, respectively. PCA scatterplots and Neighbour Joining dendrograms based on the SSR data clustered accessions into groups that more-or-less correspond to morphological diversity in both species. AMOVA within and between morphological groups revealed greater variances within groups than between. This indicates weak genetic differentiation between morphological groups, particularly for taro. Genetic diversity was greater among taro cultivars. Taro has a longer history of introduction and dispersal in Africa, and has had more opportunity for multiple introduction and local cultivar development. Different strategies are suggested for future development of these crops in Togo and Africa. For taro, further studies of existing diversity and recent experimental introductions to Africa are likely to be rewarding. New cocoyam, a modern historical introduction, has spread widely in Africa with little genetic diversity. For this crop, international collaboration is needed to clarify taxonomy, and to introduce further cultivars for evaluation under local conditions in Africa.

1. Introduction

Root and tuber crops are important sources of food and income for household in rural areas of Africa. In sub-Saharan Africa, they provide

about 20% of calories (Pinstrup-Andersen *et al.*, 1999). Taro, *Colocasia esculenta* (L.) Schott and new cocoyam, *Xanthosoma spp.*, are grown for food and income generation at the household level. Both crops are grown in tropical regions of Africa, Asia, Oceania and America, and taro is also common in temperate regions of Africa, Asia, and Oceania (Gonçalves, 2011; Matthews, 2014; Onyeka, 2014; Grimaldi, 2016; Matthews and Ghanem, 2021). Taro is considered an ancient crop in Africa, with multiple likely routes of introduction from Asia (Matthews, 2006; Fuller *et al.,* 2011; Chaïr *et al.*, 2016; Grimaldi, 2016). New cocoyam is known to have been introduced to Ghana in 1843 (Karikari, 1971), though earlier introduction following European contact with America has been suggested (Bown, 2000).

Most parts of these plants (corms, side-corms, stolons, petioles, leaf blades, and floral spathes) are edible, the leaves and corms are also commonly used as animal fodder in Asia (Coursey, 1984; Matthews, 2010; Mwenye *et al.,* 2010; Masuno *et al.*, 2012; Matthews, 2014; Wada *et al.*, 2017), and medicinal uses are also known (Plowman, 1969; Ribeiro Pereira *et al.*, 2021). The specific parts eaten vary according to cultivar attributes, local food knowledge, and cultural or personal preferences. Both crops have great potential for development in Africa and globally (Okereke, 2020).

Under cultivation, clonal propagation is universal for both taro and new cocoyam. In a global survey of taro, Chaïr *et al.* (2016) found the greatest genetic diversity and the largest number of private alleles in Asian cultivars, especially in India. Low genetic diversity was found in western Africa, among diploid and triploid cultivars, and also in southern Africa, where triploid cultivars were dominant. Their observations correspond broadly to what is known about the breeding of taro: flowering, fruiting and seed production by wild and cultivated taros are common in tropical regions of Asia and Oceania (Matthews, 2014), but have not been observed in Africa despite occasional reports of flowering (e.g. Traore, 2013). Natural fruiting and seed production by new cocoyam has not been reported outside South America, but sterile inflorescences are often produced (observation by authors), and induced flowering and experimental crosses have been reported in Cameroon (Onokpise *et al.,* 1992).

Globally, taro has undergone genetic erosion due to changes in cropping patterns, the spread of improved varieties, and replacement by other crops (Lakhanpaul *et al.*, 2003; Matthews and Ghanem, 2021), including new cocoyam (Coursey, 1984). The acridity of taro (and resulting special care needed for cooking) (Matthews, 2010), spread of taro leaf blight (TLB) (Singh *et al.,* 2012), and shortages of planting materials are contributing factors. In order to identify and preserve cultivars of economic value, maintain living germplasm collections efficiently (without excessive duplication), and provide baseline data for future breeding programs, genetic diversity and morphology must be assessed in cultivars of both species.

The taxonomy of cultivated *Xanthosoma* species and relationships with wild species are uncertain, and historically there has been a tendency to use the name *X. sagittifolium* for all cultivated *Xanthosoma* (Giacometti and Leon, 1994; Castro, 2006; Quero-Garcia *et al.*, 2010; Doungous *et al.*, 2015). Although *X. sagittifolium* is the name used in many previous studies in Africa, the plant is most likely to be *X. mafaffa* (Gonçalves, 2011). The taxonomy of cultivated *Xanthosoma* spp. in tropical America has been revised by Croat and Delannay (2017). Various cultivated species of *Xanthosoma* are also circulating internationally and may have reached Africa in the modern historical period. These include *X. atrovirens* C. Koch & Bouché (blackish green blades, and "blue" wax on dark green petioles creating a dark purple or black appearance), *X. robustum* (which can reach 4 m in height, with tall above-ground stems) and *X. violaceum* (with violet petiole tissue below a waxy surface) (Gonçalves, 2011). In addition to the 1843 introduction of *Xanthosoma* (Karikari, 1971), an unsuccessful attempt was made to introduce *X. brasiliensis* from Puerto Rico in 1974 (Karikari, 1979). In Central America, *X. mafaffa* cultivars vary in corm parenchyma color (red or white), and those with red corms are also tinged with red in the petiole, leaf sheath and spathe (Gonçalves, 2011).

In Togo, a collection of taro and new cocoyam cultivars from throughout the country was assembled, and morphological groups were described in both species (Bammite, 2018; Bammite *et al.,* 2018 a, b). However, data related to genetic diversity among these crops, based on molecular tools such as SSR, are lacking to enhance effective usage and conservation of these neglected species and develop a breeding program to improve the quality of their germplasm. This study aimed to assess simple sequence repeat (SSR) diversity in the same collection of taro and new cocoyam. Polymorphic SSR loci have codominant alleles (repeat sequences of varying length, detected by PCR amplification), and been

used in many studies of taro (Devi, 2012; You *et al.,* 2015) and other edible aroids (Suppl. Table S1), and in genetic linkage mapping of the taro nuclear genome (Quero-García *et al.,* 2010; Soulard *et al*., 2017).

2. Materials and Methods

Plant materials

In 2016, cultivars of taro and new cocoyam were collected from 42 localities randomly selected across the five ecological zones of Togo (Fig. 1). At each village, farmers were invited to bring corms of different cultivars grown in their village. For each distinct cultivar recongnized in group discussions, the local name was recorded and collected corm samples were planted at the Centre de Recherche Agronomique du Litoral (CRAL), an experimental farm of the Institut

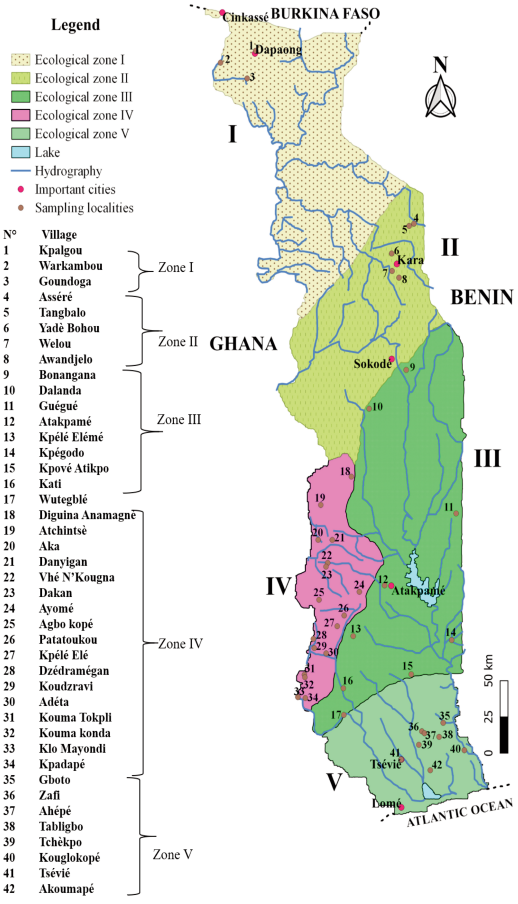

Fig. 1 - Map of Togo showing sampling localities in 2016 and ecological zones: Zone I, Northern lowlands; Zone II, Northern Togo mountains; Zone III, Central lowlands; Zone IV, Southern Togo mountains; Zone V, Coastal plains of southern Togo. Figure adopted from Bammite *et al.*, 2018 b; zones originally described by Ern (1979); base map from IGN France, 1990.

Togolais de Recherche Agronomique (ITRA) located at Davié, at latitude N 6°23' and longitude E 1°12' and at 88 m above sea level (Table S2). Accessions of both species were classified in a binary manner based on morphological characters that are easily observed in the field: taro accessions were identified as either dasheen (with large mother corms, and either stolons or side-corms; Pop1), or eddoe (with small mother corms, and few to many side-corms; Pop2); new cocoyam accessions were identified as either green (leaves entirely green; Pop1) or purple (petioles purple or pink to some extent; Pop2). Morpological diversity in the same collection was previously analysed with reference to a wide range of agronomic and morphological characters (Bammite *et al.*, 2018 b; Figs. S1-S3). Young leaf tissue from one plant from each of 26 accessions of taro and 101 accessions of new cocoyam was dried on silica gel and taken to the International Institute of Tropical Agriculture (IITA) Bioscience Centre, Ibadan, Nigeria for DNA extraction and genotyping.

DNA isolation and quantification

DNA was extracted using an optimized SDS protocol recommended by IITA Bioscience Centre (2017). About 100 mg of dry leaf tissue was put in a tube with two steel balls and reduced to powder using a SPEX Genogrinder-2000. Pre-heated extraction buffer (450 µl of 1M Tris-HCl, 0,5M EDTA, 5M NaCl, 20% SDS and 1% PVP) was added. Tubes were incubated at 65°C for 20 mins and inverted occasionally to homogenize each sample. Tubes were removed from bath, allowed to cool for two mins, then 200 µl of ice-cold 5M potassium acetate was added and the mixture incubated on ice for 20 mins to precipitate proteins. Tubes were then centrifuged at 3500 rpm for 10 mins, and each supernatant was transferred to a new labeled tube. A volume of 200 µl of 4% polyvinylpyrrolidone (PVP) was added to the supernatant and gently mixed. To precipitate and remove proteins and lipids, 45 µl of chloroform isoamylalchohol (24:1) was added, mixed gently and tubes were centrifuged at 3500 rpm for 15 mins. Each supernatant was transferred to a new tube, and a 2/3 volume of ice-cold isopropanol was added, mixed, and incubated in -80°C for 15 mins to precipitate the DNA. After centrifuging at 3500 rpm for 15 mins, the DNA pellet was washed by adding 400 µl of 70% ethanol, centrifugation at 3500 rpm for 15 mins, and decanting the supernatant until the last drop. The DNA pellet was air-dried then resuspended in 100 µl low salt TE buffer (10 mM Tris-HCl, 1 mM

EDTA). A volume of 2 µl of RNase A (10 µg/ ml) was added and incubated at 37°C for 40 mins. The quantity and quality of extracted DNA was checked using electrophoresis with 1% agarose gel, and a Nanodrop 8000 spectrophotometer, and the extracts were stored at -20°C until use.

PCR amplification

Initial testing was carried out with 47 primer pairs designed in previous SSR studies: 19 for for *Amorphophallus paeoniifolius* (Santosa *et al.,* 2007), 11 for *C. esculenta* (Hu *et al.,* 2009.) and 17 for *X. sagittifolium* (Cathebras *et al.,* 2014) (Table S3). The 47 primer pairs were tested first with five samples from each target species to determine which pairs could amplify scorable DNA products in each species.

PCR amplification was performed in PCR mixture (25 µl) containing 2.5 µl of template DNA (20ng/µl), 2.5 µl of 10x NH4 PCR reaction buffer, 1 µl of 50mM $MgCl_2$), 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer, 0.2 µl of 5mM each dNTP, 0.1 µl of BIOTAQ DNA polymerase and 16.1 µl of water. The PCR program consisted of initial denaturation (94°C, 5 mins), 42 cycles each consisting of 20 s denaturation (93°C), 1 min annealing at temperatures ranging from 47 to 59°C (as recommended by the authors above; Table S3), and 2 mins elongation (72°C). Finally, an extension period of 10 mins was included. After PCR completion, the products were stored at 4°C until gel electrophoresis. Ten µl of each PCR product was electrophoresed alongside a 50 bp DNA ladder (New England Biolab) in polyacrylamide gel (10% InstaPAGE gel) at 110 V for one hour, and bands were visualized by silver staining (1L TBE 0,5X buffer + 500 µL of SafeView) for 3 mins. Amplified fragment sizes were determined by comparison to the 50 bp ladder, and bands were examined and recorded using the ENDURO™ Gel Documentation System.

Data analysis

Recorded gel images with PCR products were analysed the the Image Studio Lite Ver 5.2 software, generating binary matrix data for all accessions based on the band patterns observed at each SSR locus: presence of an amplified band was scored as "1"; absence was scored as "0". Summary statistics for each locus were estimated using PowerMarker 3.25 software.

For statistical comparisons within each species, the binary classifications of morphotypes were used: taro - dasheen = Pop 1, eddoe = Pop2; new cocoyam - green = Pop1, purple = Pop2. GenAlex 6.5 software (Peakall and Smouse, 2006) was used to calculate the number of polymorphic loci (no. PL), the percentage of polymorphic loci (% PL), the observed number of alleles (Na), the effective number of alleles (Ne), average expected heterozygosity (He) (also known as Nei's gene diversity, Nei, 1973), and Shannon's information index (I) (a measure of genetic diversity suitable for codominant data). Analysis of molecular variance (AMOVA) was performed to evaluate the genetic variation within and among morphotype populations by using GenAlEx version 6.503 (Peakall and Smouse, 2006), and PhiPT (the proportion of total genetic variance derived from variance between individuals among populations, i.e. an estimate of population genetic differentiation). Here, our H_0 = no genetic difference among populations, H_1 = there is genetic difference among populations, and p= probability of an observed PHiPT value =/> than that observed by chance, if the null hypothesis (no genetic difference) is true.

Principal Coordinate Analysis (PCA) was carried out using the GenAlEx version 6.503 (Peakall and Smouse, 2006). The genetic distance matrix was constructed by calculating the shared allele distance for each pair of individuals in PowerMarker version 3.25. From this matrix, a neighbour joining (NJ) tree was constructed using Nei's genetic distances (Nei *et al*., 1983) in the same software.

3. Results

In the initial test with five samples of each target species, only 27 of 47 SSR primer pairs, amplified and gave polymorphic, scorable bands (Fig. 2; Table S3):

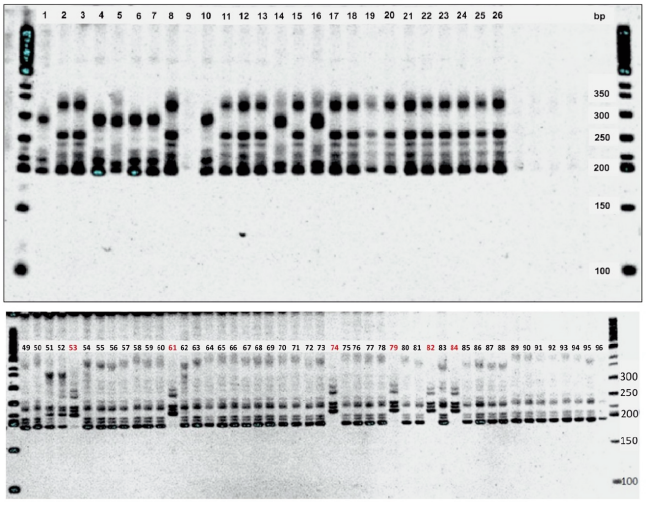

Fig. 2 - Electrophoresis of PCR amplification products to show SSR polymorphism. Above: taro tested with primer pair HK29; empty lanes at right are null results for new cocoyam tested with HK29. Below: new cocoyam tested with primer pair mXsCIR10.

two from *Amorphophallus* amplified both target species, 11 from *C. esculenta* amplified only *C. esculenta* and 14 from *X. sagittifolium* amplified only *X. mafaffa*. The resulting measures of diversity for each species are shown in Tables 1 and 4, and are summarised below.

Among 26 accessions of taro, 33 alleles were observed at 13 loci, an average of 3.15 alleles per locus (loci are hereafter identified by the primer code names). The frequency of major alleles ranged from 0.38 for locus HK7 to 0.88 for HK5, with an average of 0.62. Nei's gene diversity ranged from 0.21 for HK7 to 0.71 for HK5, with an average of 0.49. Polymorphic information content (PIC) values ranged from 0.20 to 0.65 with an average of 0.43. The primers HK35, HK26, HK38, Ampa9, HK7 with PIC values >/= 0.5 were most discriminating (Table 1). The percentages of polymorphic loci for each morphotype of taro were 82% (dasheen, Pop1) and 58% (eddoe, Pop2), with an average of 70% (rounded figures). The numbers of different (Na) and effective (Ne) alleles, Shanons Information Index (I), and Nei's gene diversity (He) were all higher in dasheen and lower in eddoe (Table 2).

Table 1 - Taro: Frequency of major alleles, number of alleles, Nei's genetic diversity, and polymorphism information content (PIC) for 13 primers applied to 26 accessions (Togo collection)

Locus	Freq. major alleles	No. alleles	Genetic diversity	PIC
HK5	0.88	3	0.21	0.2
Ampa15	0.85	3	0.27	0.26
HK25	0.77	2	0.36	0.29
HK31	0.65	2	0.45	0.35
HK29	0.65	3	0.48	0.39
AC3	0.5	3	0.54	0.43
HK22	0.65	3	0.51	0.45
HK34	0.65	3	0.51	0.45
HK35	0.65	4	0.52	0.48
HK26	0.54	3	0.59	0.52
HK38	0.42	3	0.64	0.56
Ampa9	0.42	5	0.66	0.59
HK7	0.38	4	0.71	0.65
Mean	0.62	3.15	0.49	0.43

Table 2A - Taro: Statistical measures of genetic diversity in the dasheen and eddoe populations (Togo collection)

	N	Na	Ne	I	He	%P
Pop1 (dasheen)	15	1.758	1.546	0.451	0.307	82
Pop2 (eddoe)	11	1.364	1.234	0.251	0.156	58
Mean		1.561	1.39	0.351	0.231	70
SE		0.089	0.045	0.033	0.024	12

N = no. accessions (test population), Na = no. of different alleles, Ne = no. of effective alleles, I = Shannon's Information Index, He = Nei's gene diversity, %P = percentage of polymorphic loci (rounded figures)

Table 2B - Taro: Summary analysis of molecular variation (AMOVA) in the dasheen and eddoe populations (Togo collection)

	df	SS	MS	Est. var.	% var.	PhiPT	p value
Between pops	1	15.962	15.962	0.915	17%	0.174	0.014
Within pops	24	104.23	4.343	4.343	83%		
Total	25	120.192	-	5.258	100%		

df = degrees of freedom, SS = sum of squares, MS = mean sum of squares, Est. var. = estimated variance, % var = percentage of variation, PhiPT = proportion of total genetic variance derived from variance between individuals among populations, p = probability value for PhiPT.

Among the 101 accessions of new cocoyam, 48 alleles were observed at 16 loci, an average of 3.0 alleles per locus. The frequency of major alleles ranged from 0.47 for Ampa9 to 0.97 for Ampa15, with an average of 0.83. Nei's gene diversity (He) ranged from 0.06 for Ampa9 to 0.67 for Ampa15, with an average of 0.28. PIC values ranged from 0.06 to 0.62, with an average of 0.25. Only the Ampa9 locus gave a PIC value greater than 0.5 (Table 3).

The percentages of polymorphic loci for each morphotype of new cocoyam were 74% (green, Pop1) and 94% (purple, Pop2) and with an average of 84% (rounded figures). A lower number of different alleles was recorded in the green population (Na = 1.74, n = 23) and a higher number in the purple population (Na = 1.87, n = 78), but the number of effective alleles (Ne) and other measures of diversity (I, He) were higher in the green population (Table 4).

Cluster analysis and structuring of genetic diversity

Analysis of molecular variance (AMOVA) gave percentages of molecular variance of 83% within and 17% between the dasheen and eddoe populations of taro, indicating weak differentiation overall (Table 2). For new cocoyam, the percentages of molecular variance were 64% within and 36% between the green and purple population (Table 4). The probability value (p) for PhiPT is higher in taro (0.014) than in new cocoyam (0.001) providing for taro a weaker rejection of the H_0 of no genetic difference between populations.

Table 3 - New cocoyam: Frequency of major alleles, number of alleles, Nei's genetic diversity, and polymorphism information content (PIC) for 16 primers applied to 101 accessions (Togo collection)

#	Locus	Freq. major alleles	No. of alleles	Genetic Diversity	PIC
1	Ampa15	0.97	3	0.06	0.06
2	mXsCIR1	0.95	2	0.09	0.09
3	mXsCIR1	0.94	2	0.11	0.11
4	mXsCIR0	0.89	2	0.19	0.18
5	mXsCIR1	0.87	3	0.23	0.21
6	mXsCIR1	0.87	3	0.23	0.21
7	mXsCIR1	0.85	3	0.26	0.23
8	mXsCIR2	0.86	3	0.25	0.23
9	mXsCIR2	0.85	3	0.26	0.23
10	mXsCIR2	0.84	3	0.27	0.24
11	mXsCIR1	0.83	3	0.29	0.27
12	mXsCIR2	0.79	3	0.35	0.32
13	mXsCIR0	0.76	4	0.39	0.35
14	mXsCIR2	0.77	4	0.38	0.35
15	mXsCIR1	0.76	3	0.39	0.35
16	Ampa9	0.47	4	0.67	0.62
Means		0.83	3	0.28	0.25

The first and second coordinates of the PCA scatter plot (Fig. 3) represent, respectively, 48% and 16% (in total 64%; rounded figures) of the detected variability among taro accessions. For cocoyam accessions, the coordinates represented 41% and 13% (in total 54%) (Fig. 4). Some dasheen taros formed a distinct group along the first coordinate, but apart from

Table 4A - New cocoyam: Statistical measures of genetic diversity in the green and purple populations (Togo collection)

	N	Na	Ne	I	He	%P
Pop1 (green)	23	1.742	1.532	0.418	0.287	74
Pop2 (purple)	78	1.871	1.176	0.23	0.128	94
Mean		1.806	1.354	0.324	0.208	84
SE		0.06	0.05	0.033	0.024	10

N = no. accessions tested, Na = average no. of alleles observed per locus, Ne = no. of effective alleles, I = Shannon's Information Index, He = Nei's gene diversity, %P = percentage of polymorphic loci (rounded figures).

Table 4B - New cocoyam: Summary analysis of molecular variation (AMOVA) in the green and purple populations (Togo collection)

Source	df	SS	MS	Est. var.	% var.	PhiPT	p value
Between pops	1	54.566	54.566	1.463	36%	0.36	0.001
Within pops	99	257.255	2.599	2.599	64%		
Total	100	311.822	-	4.061	100%		

df = degrees of freedom, SS = sum of squares, MS = mean sum of squares, Est. var. = estimated variance, % var = percentage of variation, PhiPT = proportion of total genetic variance derived from variance between individuals among populations, p = probability value for PhiPT.

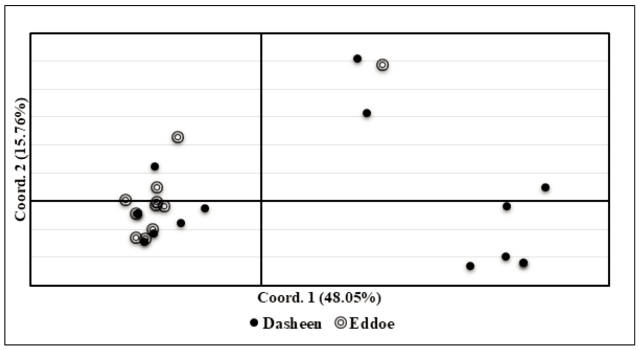

Fig. 3 - Principal coordinate analysis (PCA) of Taro accessions classified as dasheen or eddoe.

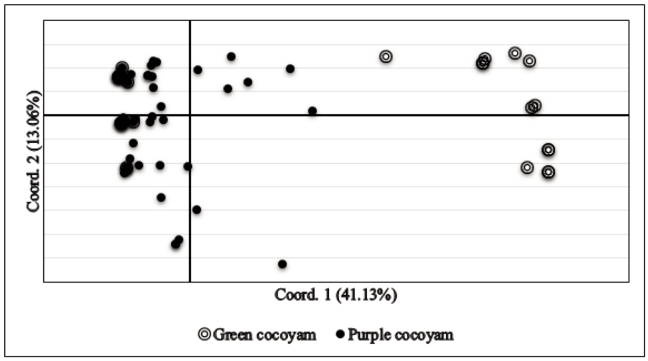

Fig. 4 - Principal coordinate analysis (PCA) of new cocoyam accessions classified as green or purple.

this, there is no clear separation of dasheen and eddoe accessions overall in the SSR data. In contrast, the green and purple new cocoyam formed very distinct groups along the first PCA coordinate. These relationships between SSR diversity in PCA scatterplot and simple morphotype classification are mirrored in the NJ dendrograms.

The NJ dendrogram of SSR diversity in taro (Fig. 5) revealed one larger cluster (C1) that includes a mix of dasheen (7 accessions) and eddoe types (10 accessions), and a two clusters (C2, C3) that include dasheen types only (9 accessions). The accessions in C3 were all dasheen types, from wet, flooded environments, with purple petiole and petiole junction, and generally producing many stolons (see Discussion and Conclusions) (Fig. S1A, Fig. S2).

The NJ dendrogram of SSR diversity in new cocoyam revealed two large clusters (C1 and C2) (Fig. 6) that largely correspond to the green and purple morphotypes of this species (13 and 88 accessions in each category; and many identical haplotypes).

With regard to the more specific morphological groups previously reported, C1 includes mostly G1, and C2 includes a mix of mostly G2 and G3 (purple morphotypes; Fig. S3).

4. Discussion and Conclusions

The present SSR results indicate few duplicate accessions in the smaller collection of taro (Fig. 5) and many apparent duplicates in the larger collection of *X. mafaffa* (Fig. 6). Qualitative and quantitative traits for morphological and agronomic characters of taro and new cocoyam were previously recorded and analysed by Bammite *et al.* (2018 b). Thirty-eight characters were selected from the descriptor list of IPGRI (1999) for *C. esculenta* and 28 from the descriptor list of IBPGR (1989) for *X. saggitifolium.* Based on these detailed observations, morphological groups within each species were identified using UPGMA analysis (Fig. S1). Although these groups (G1-

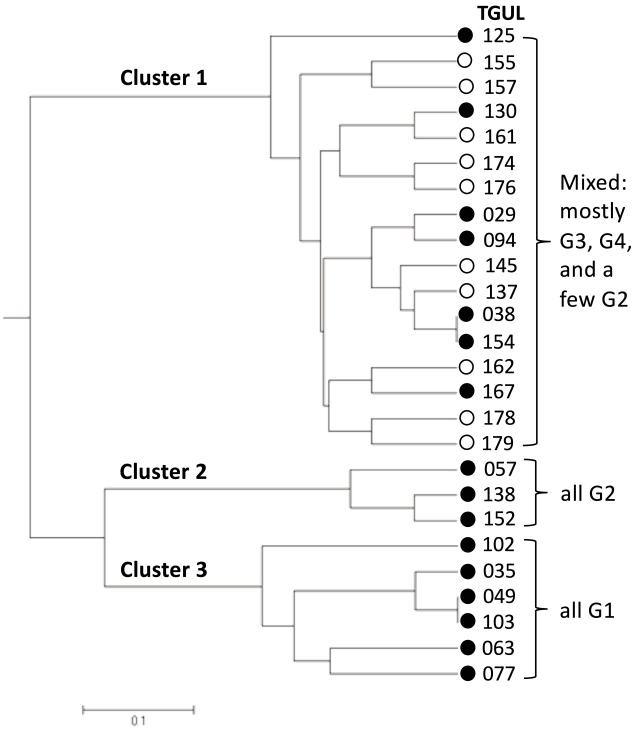

Fig. 5 - Taro: Neighbour Joining tree based on SSR data from 13 loci in 26 accessions. Cluster 1 includes both dasheen (closed circles) and eddoe (open circles) morphotypes. Clusters 2 and 3 include only dasheen morphotypes. Morphological groups identified by Bammite *et al*. (2018 b) (Figs. S1A and S2) are mixed in Cluster 1 (mostly G3, G4 and a few G2) and not mixed in Clusters 2 and 3. TGUL (Togo, University of Lomé) accession numbers are shown.

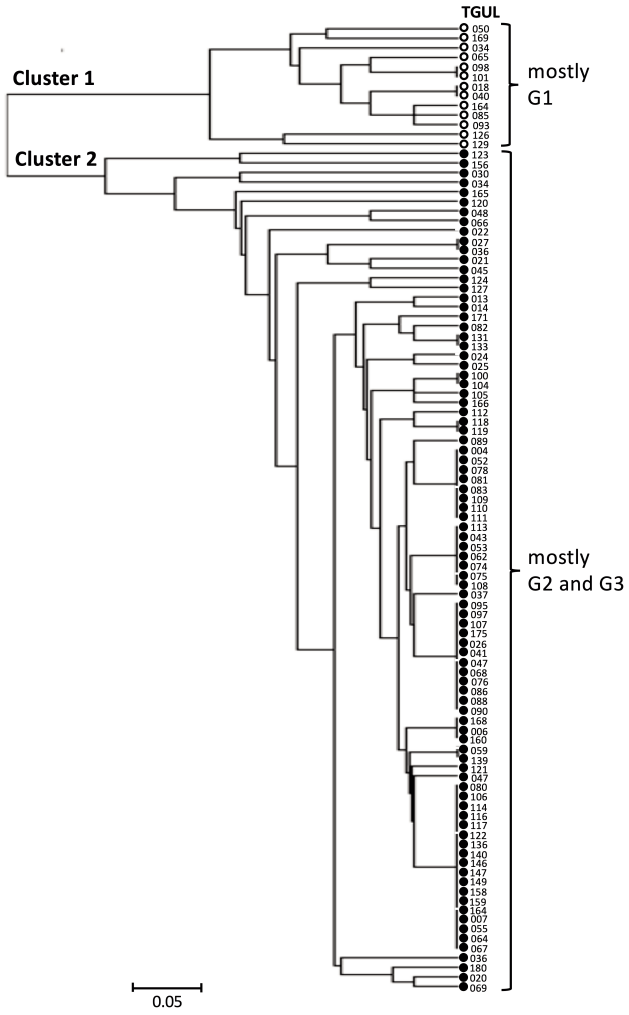

Fig. 6 - New cocoyam: Neighbour Joining tree based on SSR data from 16 loci in 101 accessions collected in Togo. Cluster 1 includes green morphotypes (open circles) and Cluster 2 includes purple morphotypes (closed circles). Morphological groups identified by Bammite *et al*. (2018b) (Figs. S1B and S3) are slightly mixed in both clusters (a few G2 and G3 in Cluster 1, and a few G1 in Cluster 2). TGUL (Togo, University of Lomé) accession numbers are shown.

G4 in taro, G1-G3 in new cocoyam) do not always correspond as expected to the single-character categories used in the present study (corm size and shape in taro, plant colour in new cocoyam) (Figs. S2 and S3), congruences are apparent between SSR genetic diversity and morphological diversity, however the latter is defined. Complete correspondence between single-character and multi-character classifications is not expected, but future studies of morphological diversity can be improved by ensuring greater uniformity in the planting materials used.

Ahmed *et al.* (2020) found that the dasheen/eddoe classification corresponds to two distinct evolutionary lineages within *C. esculenta*, and suggested that the existence of many intermediate or mixed morphotypes may reflect hybridisation between these lineages. In Togo, SSR cluster C1 includes both dasheen and eddoe forms, consistent with the suggestion of mixing (hybridisation) between eddoe and dasheen lineages (see also Lakhanpaul *et al.*, 2003). Clusters C2 and C3 corresponded entirely to dasheen forms with large mother corms and either stolons or side-corms. The three accessions in C2 had morphological above-ground traits similar to those typical of eddoe in the Togo collection (large pendant or drooping leaves) and underground morphological traits of dasheen type (G2, with large central corm with small side-corms). These accessions were collected in home gardens of farmers from Kabyè ethnic group in the ecologic zone IV (southern Togo Mountains) (Fig. 1). They reported having introduced them from ecological zone II (northern Togo Mountains), which is consistent with the report by Ern (1979) of an expansion of banana, cassava and taro production on steep slopes in Zone IV by Kabyè settlers from the north.

The observation of two major SSR clusters in new cocoyam (C1-C2) suggests that more than one species of *Xanthosoma* is present in Togo, not just *X. mafaffa* (Bammite *et al.,* 2018 a, b). A survey of Amplified Fragment Length Polymorphism (AFLP) in new cocoyam in Ethiopia also revealed two major clusters (Wada *et al.*, 2018). The existence of such distinct lineages and the general uncertainty of identification of *Xanthosoma* species suggest an urgent need for direct and detailed comparisons between cultivated *Xanthosoma* spp. in Africa, and the wild and cultivated species of *Xanthosoma* in tropical America.

For both species, the Neighbour Joining analysis of SSR allelic diversity at a small number of loci provides tree diagrams in which terminal branching (near tree tips) is not a reliable indicator of phylogeny. Much of the "within population" variation in both species may reflect somatic mutation within clonal cultivar lineages. The small numbers of loci analysed here (13 in taro, 14 in new cocoyam) make it inherently difficult to distinguish clones with certainty, as there are 14 chromosomes in the haploid complement of *C. esculenta* (Coates *et al.*, 1988; Cusimano *et al.,* 2012), and 13 in *Xanthosoma* spp. (Cusimano *et al.*, 2012; Wada *et al.*, 2018) giving rounded averages of just 0.9 loci (taro) and 1.1 loci (new cocoyam)

sampled per chromosome. For taro, actual coverage is less than 0.9 loci/chromosome, as two HK loci have been mapped to one linkage group and chromosome (Table S4). Although Chaïr *et al.* (2016) introduced a method to estimate clonality based on just 11 loci, the same research group also employed a much more robust method using Diversity Array Technology (DArT™) to screen polymorphic loci (possibly thousands) across the entire taro genome (Vandenbrouke *et al.*, 2016). By combining the latter method with a detailed survey of morphological diversity in an assemblage of Vanuatu cultivars, Vandenbrouke *et al.* (2016) could unequivocally identify clonal lineages within which somatic mutation has produced distinct phenotypes that are recognised, selected, and maintained by farmers. Most recently, Soulard *et al.* (2017) have mapped polymorphic SNP and SSR loci across the entire taro nuclear genome, while Yin *et al.* (2021) have published nearly complete sequences for all 14 chromosomes in taro.

Somatic mutation may explain some of the morphological and genetic diversity found in taro in Togo and Africa generally, but spontaneous breeding among diploid cultivars in Africa may also be involved. The chromosome numbers of Togo cultivars have not been studied, but triploid taros are widespread and common in Africa (Chaïr *et al.*, 2016) and the neighbouring country of Burkina Faso (Traore, 2013), and can be assumed to be inherently sterile because they are triploids. The eddoe-type taros (*C. esculenta* var. *antiquorum*) in Togo are likely to be triploids, as this morphotype is generally triploid in neighbouring Burkina Faso (Traore, 2013) and eastern Asia (Plucknett, 1983; Matthews, 2014; Wang *et al*., 2020), but this cannot be assumed - if the diploid (fertile) progenitors of triploid eddoe cultivars still exist, some might share the eddoe morphotype. It also cannot be assumed that the dasheen types are diploid. There are multiple triploid lineages in taro, and some dasheen and intermediate morphotypes are also triploid (Kreike *et al*., 2004).

The results of our initial primer screening corroborate those of Traore (2013), who found that primers designed for *C. esculenta* are not transferable to *Xanthosoma spp.* accessions. We also found that, conversely, the primers designed for *Xanthosoma* do not amplify *C. esculenta* accessions. In their original report of the HK primers, Hu *et al.* (2009) surveyed 30 plants from several provinces of China. Chaïr *et al.* (2016) screened 64 primer pairs developed from *C. esculenta* and *A. paeoniifolius*, and selected 11 from *C. esculenta*, of which three were from the HK primer series. This study (the largest survey of SSR diversity in taro) included 321 cultivars from 19 countries in Asia, Africa, America and the Pacific. Several HK primers were also used by Hunt *et al.* (2013). Including the present Togo survey, results for HK7, HK22 and HK26 can now be compared across four studies (Table 5). The largest number of alleles was found in the largest sample set representing many countries (Chaïr *et al.*, 2016), which is not surprising. The surprise here is that Togo, a relatively small country far from Asia, displayed only slightly fewer alleles than a similar number of plants from across China (Hu *et al.*, 2009), a much larger country that is also a candidate region for the origin of triploid taros (Matthews, 2014; Wang *et al.,* 2020; Zhu *et al.*, 2000). The number of alleles in two wild breeding populations in Papua New Guinea and an adjacent region of northern Australia was larger than in the China and Togo cultivars, but also much less than in the large survey by Chaïr *et al.* (2016).

The relatively low number of alleles detected in Togo presumably reflects the small number of plants

Table 5 - Sample size (n) and number of alleles at SSR loci in Taro, in four different studies using the HK primer series designed by Hu *et al*. (2009)

Locus	Number of alleles			
	Hu *et al.* (2009), n=30, China	Hunt *et al.* (2013), n=42–49, Australia & PNG (two wild populations)	Chaïr *et al.* (2016), n= 321 (19 countries excl. China)	Present study n= 26, Togo
HK5	6	10	-	3
HK7	4	2	12	4
HK22	3	-	18	3
HK26	5	8	28	3
HK31	3	4	-	2
HK34	3	9	-	3
HK35	3	11	-	4

(-) = loci not studied

tested, and the relatively low genetic diversity of taro in Africa generally (Chaïr *et al.* 2016). Nevertheless, the overall diversity of taro in Togo, and in the neighbouring countries of Ghana and Burkina Faso (Traore, 2013) does suggest a complex history of the crop in the region, and in Africa.

Among published studies of SSR diversity in taro (Table S1), no two studies have used the same methods to collect, maintain and test plants, and no standard set of primer pairs and target loci has emerged. Crucially, different sample sets differ in whether they represent initial collections created to assess diversity in possibly-identical cultivars from different locations (as in the present study), or later-stage collections in which apparent duplicates have been removed. Observed diversity depends on how plants are collected, how many are collected, and how each collection is maintained over time. Taro and new cocoyam collections are constructed "populations" of clones, not random samples from freely breeding populations. For all of these reasons, we do not compare our statistical estimates (calculated data) with those of other small-scale studies. In the near future, new techniques for large-scale and low-cost DNA sequencing may allow more accurate, comprehensive and direct comparison of genotypes in different cultivar assemblages. Already for taro, public databases contain records of thousands of SSR and SNP (single nucleotide polymorphism) loci revealed by whole-genome and transcriptome studies (Liu *et al.,* 2015; You *et al.*, 2015; Helmkampf *et al.,* 2017; Soulard *et al.,* 2017; Wang *et al.*, 2020), and a draft sequence for all 14 chromosomes has been published (Yin *et al.*, 2021).

In the Togo collection, flowering occurred among accessions of both species, but fruiting and seed production were not observed. Togo has a tropical savannah climate, with distinct wet and dry seasons, and annual rainfall ranging from around 800 mm to 1,600 mm (Djaman *et al.*, 2017). In Burkina Faso, in the same general climate zone, but further Northwest, most taro is mostly grown in provinces with annual rainfall ranging from around 700 mm to 1,100 mm, near Togo (Traore, 2013). Although conditions during the wet season in Togo (April to October) might be suitable for breeding by taro, dry and windy conditions during the winter harmattan (Ern, 1979) would be fatal for unprotected seedlings. These are very different circumstances from those in the natural range of taro, in the tropical rainforest zone of Asia and the western Pacific, where wild breeding populations are found (Matthews, 1991; Hunt *et al.*, 2013; Matthews, 2014). Togo itself lies in a dry savannah corridor (the Dahomey Gap) flanked by tropical rainforest (the Upper and Lower Guinean Forests) where spontaneous breeding by taro may be possible. Breeding and selection of taro cultivars in these nearby forest regions might have contributed to some of the diversity found in Togo.

Different strategies are suggested here for future development of taro and new cocoyam in Togo. For taro, it will be rewarding to study the existing range of eddoe and dasheen cultivars further, and to make experimental introductions of new cultivars from outside Africa, following the example of Ouedraogo *et al.* (2018). Efforts will be needed to produce disease-free stocks of existing cultivars so that fair comparisons can be made with newly-introduced plants that are disease free. New cocoyam, a relatively modern historical introduction, has spread widely in Africa, and a lack of diversity is clear among the accessions collected in Togo. International collaboration is needed to identify and introduce new cultivars for evaluation under local conditions. This will be difficult, as there are no international breeding programmes for the crop, and little is known about the origins and diversity of cultivated *Xanthosoma* species in Central and South America. *Xanthosoma* spp. are even more neglected as orphan crops than taro (Matthews and Ghanem, 2021).

Together, taro and new cocoyam offer a range of cultivars suitable for cultivation in wetland to dryland environments. Agriculture in Togo is predominantly rainfed and often experiences both flooding and drought (Djaman *et al.*, 2017). In seasons and locations when water is abundant, the flooding tolerance of dasheen taro (Onwueme, 1999) is a positive attribute that can enhance the food security of farmers working in riverine flood plains. When irrigation is provided, very good yields of taro can be expected in otherwise dry environments: this is shown by the success of taro as an irrigated summer crop in the eastern Mediterranean (Matthews, 2006), a region with long dry summers and relatively little annual rainfall (approx. 400 mm per year in the main agricultural districts of Cyprus). Over the last 50 years, climate change has been very obvious throughout Togo, with the wet season becoming 1-2 months shorter (Djaman *et al.*, 2017; Gadédjisso-Tossou, 2018). During the period 1961-2001, for example, annual precipitation decreased at 80% of weather stations across the country (Djaman *et al.*, 2017).

Under these circumstances, maintaining or expanding the cultivation of taro and new cocoyam may come to depend on the success or otherwise of efforts to improve methods for water storage, conservation and irrigation in Togo.

Acknowledgements

The authors greatly appreciate the International Foundation for Science (IFS Research Grant No. C/5866-2, 2020-2023) for providing research funds. The contribution by Matthews was partly funded by JSPS Kakenhi No. 17H04614 (2017–2020). Thanks also to E. Tabuchi, Osaka, for assistance with figures and tables.

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Supplementary materials

Genetic diversity in *Colocasia esculenta* and *Xanthosoma mafaffa* in Togo, West Africa

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Citation:
BAMMITE D., MATTHEWS P.J., DAGNON D.Y., AGBOGAN A., AGRE P., AKINTAYO T.O., ODAH K., DANSI A., ABBERTON M., TOZO K., 2021 - *Genetic and morphological diversity in Colocasia esculenta and Xanthosoma maffafa in Togo, West Africa.* - Adv. Hort. Sci., 35(3): 255-267

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 9 September 2020
Accepted for publication 9 July 2021

Key words: crop diversity, new cocoyam, SSR, Taro, Togo.

Abstract: Taro and new cocoyam are root and leaf crops commonly grown in tropical to warm temperate regions. In Togo, they are neglected and underutilized. Here we report the genetic and morphological diversity of 26 accessions of taro and 101 accessions of new cocoyam. Analysis of simple sequence repeats revealed low polymorphic information content of 0.43 and 0.25 in taro and new cocoyam, respectively. PCA scatterplots and Neighbour Joining dendrograms based on the SSR data clustered accessions into groups that more-or-less correspond to morphological diversity in both species. AMOVA within and between morphological groups revealed greater variances within groups than between. This indicates weak genetic differentiation between morphological groups, particularly for taro. Genetic diversity was greater among taro cultivars. Taro has a longer history of introduction and dispersal in Africa, and has had more opportunity for multiple introduction and local cultivar development. Different strategies are suggested for future development of these crops in Togo and Africa. For taro, further studies of existing diversity and recent experimental introductions to Africa are likely to be rewarding. For new cocoyam, a modern historical introduction, has spread widely in Africa with little genetic diversity. International collaboration is needed to clarify taxonomy, to select and introduce further cultivars for evaluation under local conditions in Africa.

Table S1 - Annotated bibliography of simple sequence repeat (SSR) studies in Araceae. Reference details are provided at the end of supplementary materials

References	Target taxa	Primers developed/used
Amorphophallus		
Santosa *et al.*, 2007	*A. paeoniifolius*, cultivars	***Ampa*** primer series first reported
Santosa *et al.*, 2010	*A. paeoniifolius,* cultivars	Used *Ampa* primers; geographical survey
Pan *et al.*, 2012	*A. konjac*	***Amor*** primer series first reported
Colocasia esculenta		
Mace and Godwin, 2002	Cultivars; Asia and Pacific	***uQ*** primer series first reported (later authors refer to these with *Xqutem* prefix)
Noyer *et al.*, 2003	Cultivated and wild; Asia and Pacific	***Ces*** primer series first reported (did not amplify *X. sagittifolium*)
Mace *et al.*, 2006	Cultivars; Pacific	*Xqutem (uQ)* primer series used to reduce duplication in large regional collection
Singh *et al.*, 2007	Cultivars; Papua New Guinea	*Xqutem (uQ)* primers used to reduce duplication in living cultivar collection
Hu *et al.*, 2009	Cultivars; China	***AC-HK*** primer series first reported, used for geographical survey
Quain *et al.*, 2010	Cultivars; Ghana	First report: **Sweet potato EST-SSR** primers successful in test with taro cultivars
Mace *et al.*, 2010	Cultivars; Pacific	*Xqutem (uQ)* primer series used to reduce duplication in large regional collection
Lu *et al.*, 2011	Cultivars; China	***Taro*** series first reported; geographical survey
Sardos *et al.*, 2012	Cultivars; Asia and Pacific	*Ces* primers used for geographical survey
Hunt *et al.*, 2013	Wild; Papua New Guinea and northern Australia	*uQ a*nd *AC-HK* primers used to survey wild breeding populations
Mabhaudhi and Modi, 2013	Cultivars; South Africa	*uQ* primers used to test dasheen and eddoe morphotypes; geographical survey
Traore, 2013	Cultivars; Burkina Faso	Results later published in Chaïr *et al.*, 2016 (see below)
Macharia *et al.*, 2014	Cultivars; Kenya, Tanzania, Uganda	*Xqutem (uQ)* primers used for Lake Victoria regional survey
You *et al.*, 2015	Wild and cultivated; China (36% of primer pairs succeeded with *Alocasia macrorrhizos)*	Thousands of EST-SSR loci detected, ***CE-EST-SSR*** primer series first reported; wild taro in Jiangxi a distinct clade
Chaïr *et al.*, 2016	Mostly cultivars; Asia, Africa, Oceania, America	*Ces*, *uQ*, and *HK* primers used; geographical survey, 19 countries
Dai *et al.*, 2016	Cultivars; China	*Ces* and *Taro* primers used to develop cultivar-specific SSR-SCAR test; coupled with chloroplast DNA
Palapala and Akwee, 2016	Cultivars; Kenya	*Xqutem (uQ)* primers used; geographical survey
Rasco *et al,* 2016	Cultivars, Philippines	Used *uQ* primers, ***CT*** primers from cassava (Moyib *et al.*, 2007), and ***SSRY*** primers from citrus (no
Mezhii *et al.*, 2017	Cultivars, India	***COL*** primer series first reported here (designed by GCC Biotech, Kolkata); geographical survey
Soulard *et al.*, 2017	Experimental crosses (cultivar breeding lines), Vanuatu	Loci detected by *uQ, Ces, HK,* and *Taro* (FJ895341) primers mapped (alongside SNP loci) in 14 linka-
Khatemenla *et al.*, 2019	Cultivars, India	*uQ*, and ***Ce1*** primers used; geographical survey (no ref. cited for Ce1)
Xanthosoma spp.		
Traore, 2013	*X. sagittifolium*, *X. robustum*, *X. mafaffa*, *X. dealbatum, X. mexicanum,* from Costa Rica and India	*Ces*, *uQ*, and *HK* primer series used, with some success (part of the study with Chaïr *et al.,* 2016); taxonomic survey
Cathebras *et al.,* 2014	*X. atrovirens, X. blandum, X. brasiliense, X. ceronii, X. granvillei, X. harlingii, X. hylaeae, X. mexicanum, X. piquambiense, X. poeppigii, X. pubescens, X. robustum, X. sagittifolium, X. violaceum, X. viviparum*, from central and South America	***Xs*** primer series first reported; taxonomic survey

Table S2 - List of 127 accessions of taro and new cocoyam in the Togo collection

Sample ID	Morphological groups	Species	Local name	Village
TGULT047	G3	*X. mafaffa*	Mancani Djin	Kouma konda
TGULT047 bis	G1	*X. mafaffa*	Mancani Djin	Kouma konda
TGULT048	G1	*X. mafaffa*	Mancani Hé	Koudzravi
TGULT050	G1	*X. mafaffa*	Mancani Hé	Dzédramégan
TGULT052	G3	*X. mafaffa*	Mancani Hé	Dzédramégan
TGULT053	G3	*X. mafaffa*	Mancani Djin	Dzédramégan
TGULT055	G3	*X. mafaffa*	Mancani Djin	Dzédramégan
TGULT059	G3	*X. mafaffa*	Manguélé moédé	Vhé N'Kougna
TGULT062	G3	*X. mafaffa*	Manguélé moédé	Vhé N'Kougna
TGULT064	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT065	G1	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT066	G1	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT067	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT068	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT069	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT074	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT075	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT076	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT078	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT080	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT081	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT082	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT083	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT085	G1	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT086	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT088	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT089	G2	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT090	G3	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT093	G1	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT095	G3	*X. mafaffa*	Mancani Djin	Kpélé Elémé
TGULT097	G3	*X. mafaffa*	Manguélé moédé	Aka
TGULT098	G1	*X. mafaffa*	Manguélé kpèlè	Aka
TGULT100	G3	*X. mafaffa*	Mancani Djin	Kpélé Elé
TGULT101	G1	*X. mafaffa*	Mancani Hé	Kpélé Elé
TGULT104	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT105	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT106	G3	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT107	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT108	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT109	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT110	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT111	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT112	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT113	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT114	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT116	G2	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT117	G3	*X. mafaffa*	manguélé kpèlè	Danyigan
TGULT118	G2	*X. mafaffa*	manguélé kpèlè	Danyigan
TGULT119	G3	*X. mafaffa*	Manguélé moédé	Danyigan

to be continued..

Table S2 - List of 127 accessions of taro and new cocoyam in the Togo collection

Sample ID	Morphological groups	Species	Local name	Village
TGULT047	G3	*X. mafaffa*	Mancani Djin	Kouma konda
TGULT047bis	G1	*X. mafaffa*	Mancani Djin	Kouma konda
TGULT048	G1	*X. mafaffa*	Mancani Hé	Koudzravi
TGULT050	G1	*X. mafaffa*	Mancani Hé	Dzédramégan
TGULT052	G3	*X. mafaffa*	Mancani Hé	Dzédramégan
TGULT053	G3	*X. mafaffa*	Mancani Djin	Dzédramégan
TGULT055	G3	*X. mafaffa*	Mancani Djin	Dzédramégan
TGULT059	G3	*X. mafaffa*	Manguélé moédé	Vhé N'Kougna
TGULT062	G3	*X. mafaffa*	Manguélé moédé	Vhé N'Kougna
TGULT064	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT065	G1	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT066	G1	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT067	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT068	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT069	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT074	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT075	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT076	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT078	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT080	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT081	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT082	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT083	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT085	G1	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT086	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT088	G3	*X. mafaffa*	Manguélé moédé	Dakan
TGULT089	G2	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT090	G3	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT093	G1	*X. mafaffa*	manguélé kpèlè	Dakan
TGULT095	G3	*X. mafaffa*	Mancani Djin	Kpélé Elémé
TGULT097	G3	*X. mafaffa*	Manguélé moédé	Aka
TGULT098	G1	*X. mafaffa*	Manguélé kpèlè	Aka
TGULT100	G3	*X. mafaffa*	Mancani Djin	Kpélé Elé
TGULT101	G1	*X. mafaffa*	Mancani Hé	Kpélé Elé
TGULT104	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT105	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT106	G3	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT107	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT108	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT109	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT110	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT111	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT112	G2	*X. mafaffa*	Manguélé kpèlè	Danyigan
TGULT113	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT114	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT116	G2	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT117	G3	*X. mafaffa*	manguélé kpèlè	Danyigan
TGULT118	G2	*X. mafaffa*	manguélé kpèlè	Danyigan
TGULT119	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT120	G3	*X. mafaffa*	Manguélé moédé	Danyigan

to be continued..

Table S2 - List of 127 accessions of taro and new cocoyam in the Togo List of 127 accessions of taro and new cocoyam in the Togo collection

Sample ID	Morphological groups	Species	Local name	Village
TGULT120	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT121	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT122	G3	*X. mafaffa*	Manguélé moédé	Danyigan
TGULT123	G3	*X. mafaffa*	Mancani Djin	Ayomé
TGULT124	G3	*X. mafaffa*	Mancani Djin	Ayomé
TGULT127	G3	*X. mafaffa*	mancani	Guégué
TGULT128	G1	*X. mafaffa*	Mancani koulomo	Kpélé Elémé
TGULT129	G1	*X. mafaffa*	Mancani koussèmo	Kpélé Elémé
TGULT131	G3	*X. mafaffa*	Mancana	Kpégodo
TGULT133	G3	*X. mafaffa*	Mancani Djin	Kpové Atikpo
TGULT136	G3	*X. mafaffa*	Bancani	Dalanda
TGULT139	G3	*X. mafaffa*	Bancani	Dalanda
TGULT140	G3	*X. mafaffa*	Bancani	Bonangana
TGULT146	G3	*X. mafaffa*	Bancani	Welou
TGULT147	G2	*X. mafaffa*	Bancani	Welou
TGULT149	G3	*X. mafaffa*	Bancani	Asséré
TGULT156	G3	*X. mafaffa*	Mancani Djin	Atchintsè
TGULT158	G3	*X. mafaffa*	Mancani	Atchintsè
TGULT159	G3	*X. mafaffa*	Mancani pibal	Diguina Anamagnè
TGULT160	G3	*X. mafaffa*	Mancani pibal	Diguina Anamagnè
TGULT164	G1	*X. mafaffa*	Bancani	Warkambou
TGULT164bis	G3	*X. mafaffa*	Bancani	Warkambou
TGULT165	G1	*X. mafaffa*	Bancani	Warkambou
TGULT166	G3	*X. mafaffa*	Bancani	Goundoga
TGULT168	G3	*X. mafaffa*	Bancani	Kpalgou
TGULT169	G3	*X. mafaffa*	Bancani	Kpalgou
TGULT171	G3	*X. mafaffa*	Bancani	Yadè Bohou
TGULT175	G3	*X. mafaffa*	Bancani	Yadè Bohou
TGULT180	G3	*X. mafaffa*	Bancani	Yadè Bohou

Table S3 - Characteristics of 47 SSR primers tested and recommended annealing temperatures. Sources for the primer sequences were: Ampa series = *Amorphophallus paeonifolius* (Santosa *et al.*, 2007), AC-HK series = *Colocasia esculenta* (Hu *et al.*, 2009), Xs series = *Xanthosoma sagittifolium* (Cathebras *et al.*, 2014)

#	Primer pair ID	Repeat motif	Forward (F) and reverse (R) primer sequences	Average annealing T °C	*Used/not used
1	Ampa01	(CT)5(GT)10	F: GAGTCTACGATCTGCGACTTC R: CACCAATACAACATATGTGTG	52.1	ns
2	Ampa02	(CT)13(GT)7	F: CACCCGATTGCGTTGTGCACT R: TCTCCCTTCCTACTCATCCAC	58.2	ns
3	Ampa03	(CT)7(CA)9 (TG)16	F: GATTTAGAAAGCTGGCTAGGG R: CCAGCATCCAGATGATCATC	53	ns
4	Ampa04	(CT)7(GT)10	F: CCTCTGTCACAGGTTTAGTAC R: AGCCCCAAGTACAAAGCTGG	55	ns
5	Ampa05	(TC)19(TG)10	F: CCTCCCTCTAAGTGATCAAGG R: GAGATATAAGGGTTGAAGTTC	51	ns
6	Ampa06	(TG)18(AG)9	F: GAACCTACACCGTGAGGAAAATGTTGG R: GGTGTTGAGCTAGGCCAATAC	57.6	ns
7	Ampa07	(TG)11(AG)15	F: GCTTTCAAGAGTCTCCTACTATCTAAC R: CTCTCCGTCCAGAGATGCAAC	55.9	ns
8	Ampa08	(TG)18(AG)7	F: GCGTTCTCTCAGGATAAATCCACCAAC R: GAAGCCGTAGCCCTAAGAAG	57.2	ns
9	Ampa09	(TG)11(AG)14	F: CCAAACCAATCACGCCTCAG R: GACCAAAATACCCTCATTGAC	54	Ce+ Xm+
10	Ampa10	(TG)13(AG)15	F: CGAGTCCAATCTGAACCTAATTCATTG	55	ns

Primers were (a) used, or (b) not used as follows. (a) used to test all samples in full survey (30 primer pairs): Yellow highlight, Ce+, Xm+ = polymorphic in taro and new cocoyam (2 pairs) (scorable in both species). Green highlight, Ce+ = polymorphic in taro (11 pairs); not scorable in new cocoyam. Grey highlight, Xm+ = polymorphic in nex cocoyam (14 pairs); not scorable in taro. No highlight, mmXm= monomorphic in new cocoyam (3 pairs); not scorable in taro.(b) No used in full survey (17 primer pairs): not scorable (ns) in taro or new cocoyam.

to be continued..

Table S3 - Characteristics of 47 SSR primers tested and recommended annealing temperatures. Sources for the primer sequences were: Ampa series = *Amorphophallus paeonifolius* (Santosa *et al*., 2007), AC-HK series = *Colocasia esculenta* (Hu *et al*., 2009), Xs series = *Xanthosoma sagittifolium* (Cathebras *et al*., 2014)

#	Primer pair ID	Repeat motif	Forward (F) and reverse (R) primer sequences	Average annealing T °C	*Used/not used
11	Ampa11	(TC)6(TG)14	F: CATGCGCCTTGTGGCACTCAC R: CACCATAGCCATTCACCTTG	57.7	ns
12	Ampa12	(TG)11(AG)10	F: GAGATAGAGAGAGATAAGAGTG R: CTTGAAAATCCTTACAATAGT	47.1	ns
13	Ampa13	(TG)7 … (TG)24(AG)13	F: CACTATGTGCCTTTGTAAATGGGGCAG R: CCGTCTACTCGTATGTATAC	54.45	ns
14	Ampa14	(CT)20	F: CTGTACACATCTCTTTCCACTTTATAG R: CCTGACTAAATAAATCCAGTG	50.75	ns
15	Ampa15	(GA)7(GT)11	F: CACCTTGACCGTACGAGAGAC R: CAAGCTGTAGCTAGAGAGTG	54.55	Ce+ Xm+
16	Ampa16	(AG)10(TG)7	F: CGAGTGTGGCATAGCATAGCA R: GGACTTTGCGTTCCTCACGAC	57.9	ns
17	Ampa17	(AG)12(TG)9(AG)3	F: GAAAGAAAGGCAAATAGCAGG R: CTCATCAGTTACCCCCTCCCT	55	ns
18	Ampa18	(CT)17	F: GCATTTTATGACATTGAATCCATTAAG R: GCGATCGCTCACGCGTGAG	56.65	ns
19	Ampa19	(GA)7(GT)8 (CG)6 … (CT)11	F: GCTCGCACGCACAGAAAGAG R: CTCACCGGTATACGAGTGAC	56.75	ns
20	AC3	(GT)8(AG)9	F: AGTGGCATCAATGGAGGA R: CCACTAAACGACGACCCAC	54.55	Ce+
21	HK5	(AG)28	F: CCCACCTCTTCCCATTCGCTT R: CGATCCTTCCAGCTCCGACAT	59.3	Ce+
22	HK7	(CT)14TTCTT(CT)4	F: GTTGTCCGCCTGTGCGTTCT R: CTCTTGGGAATTCTCCGGGTG	59.2	Ce+
23	HK22	(AG)18	F: ACATCAAACCTCTGGTGGGC R: AGCAATCCTAGCCGAGGTG	57.4	Ce+
24	HK25	(AC)22	F: TGACTAGGCAGGAAGGTAA R: CAAGCATTCTCTGAACTATG	50.5	Ce+
25	HK26	(CT)15	F: GGGTGTTATCGCCATAGTCAT R: GAAACACCACAACGGAGAAAC	54.5	Ce+
26	HK29	(CT)42	F: GTCTGTGGAACCCTCAAGC R: ATTGTGGGAGCGATAGGG	55	Ce+
27	HK31	(GT)6(GA)11	F: TACCGCCGAGTGCTTATC R: TACGGCTGGAATCAAAGC	53.5	Ce+
28	HK34	(AG)29	F: TTACTCCAAACGAGGCAAAC R: CCTTCAAGATGTTACCAAATGC	52.7	Ce+
29	HK35	(CT)15L(CT)9	F: TACTAGAACCCCGTCAGTCT R: CGTCGATTTATCAGTGAGC	52.5	Ce+
30	HK38	(AG)12	F: AAACGCGGCCAGAAGATC R: GAATAGCGGAACAAGGTAGA	53.7	Ce+
31	mXsCIR05	(CA) 8 (CACA) 3	F: GCGCATTATTAACGAATATC R: GTCATCTATGGCTATCACCT	49	Xm+
32	mXsCIR07	(TG) 7 (AG) 19	F: GGACTGGGAGTCTGAGTAG R: CCTTTCCCCTCACTATAAA	51	Xm+
33	mXsCIR10	(AG) 22	F: GATGTCTGTAGTGGCCTAGT R: AATTAAGTTGGGTGGTAGAT	51	Xm+
34	mXsCIR11	(TG) 10 (GA) 16	F: AATTCTTAGCAGCATTGTTA R: CATTCGTATCAACTTCCTTT	47.6	Xm+
35	mXsCIR12	(TC) 17 (TTC) 7 (TCCC) 3 (TTCTTG) 3	F: TACATTTCCATTGCCATC R: CAAATTAAAGAGGGAGACAG	47.7	Xm+

Primers were (a) used, or (b) not used as follows.
(a) used to test all samples in full survey (30 primer pairs): Yellow highlight, Ce+, Xm+ = polymorphic in taro and new cocoyam (2 pairs) (scorable in both species). Green highlight, Ce+ = polymorphic in taro (11 pairs); not scorable in new cocoyam. Grey highlight, Xm+ = polymorphic in nex cocoyam (14 pairs); not scorable in taro. No highlight, mmXm= monomorphic in new cocoyam (3 pairs); not scorable in taro.
(b) No used in full survey (17 primer pairs): not scorable (ns) in taro or new cocoyam.

to be continued..

Table S3 - Characteristics of 47 SSR primers tested and recommended annealing temperatures. Sources for the primer sequences were: Ampa series = *Amorphophallus paeonifolius* (Santosa *et al*., 2007), AC-HK series = *Colocasia esculenta* (Hu *et al*., 2009), Xs series = *Xanthosoma sagittifolium* (Cathebras *et al*., 2014)

#	Primer pair ID	Repeat motif	Forward (F) and reverse (R) primer sequences	Average annealing T °C	*Used/not used
36	mXsCIR13	(CA) 8 (AG) 16	F: GTTTCCTTTATTCGTTGATG R: GTAGTGGCTGAGAATTGAAA	48.6	Xm+
37	mXsCIR14	(AG) 20	F: TACCCTACATTTGGGATCT R: TTTTGGCTTTAGGTCTATTC	48.7	Xm+
38	mXsCIR16	(AG) 15	F: CTTATTGATGCCGAGAATAC R: TTCCTCACAATATGTTCTCAT	48.6	Xm+
39	mXsCIR19	(AC) 8 (AC) 24 (AC) 8	F: CAACTTGTGTATCCTACATCC R: GCGTGGTTTATGTGTATCTT	50.5	Xm+
40	mXsCIR20	(CT) 11 (TC) 15 (TCTA) 3	F: CCCTTATTGCTGTTTTCA R: CATATCTCTTCCTCTCACCA	49	Xm+
41	mXsCIR21	(AG) 30	F: CTTAACCTTGTCAGCCTCT R: GAGCGGTATAACAATTCATC	50.1	Xm+
42	mXsCIR22	(AG) 22	F: CGTGAGAAACACCTGAATTA R: AATTTGCTCTGTCATTGTG	49.3	Xm+
43	mXsCIR23	(GA) 23	F: TGTAGGTATGGACACATGG R: TTAAGACAAACCCTCAGC	50.2	mmXm
44	mXsCIR24	(AG) 23	F: AATTTGAAGTGAAACGATCA R: TTCCTGTCATCAGAATTGTA	48.1	Xm+
45	mXsCIR26	(TC) 9 (TC) 9	F: TTCACCATTACTTGTCCACT R: TTAACATGGGAACGTATCTT	50	mmXm
46	mXsCIR27	(AG) 15 (GAA) 6	F: TGCATGAATTGAAGAAAT R: AACAAAGAGTCTCACCACAT	47.9	Xm+
47	mXsCIR28	(GA) 9	F: ACAGAAGTTGACATGGAGAG R: AATGTTAAAGAGCAAAAGGA	49.7	mmXm

Primers were (a) used, or (b) not used as follows.
(a) used to test all samples in full survey (30 primer pairs): Yellow highlight, Ce+, Xm+ = polymorphic in taro and new cocoyam (2 pairs) (scorable in both species). Green highlight, Ce+ = polymorphic in taro (11 pairs); not scorable in new cocoyam. Grey highlight, Xm+ = polymorphic in nex cocoyam (14 pairs); not scorable in taro. No highlight, mmXm= monomorphic in new cocoyam (3 pairs); not scorable in taro.
(b) No used in full survey (17 primer pairs): not scorable (ns) in taro or new cocoyam.

Table S4 - Mapped locations of taro SSR loci detected by the HK primer series1. HK7 and HK26 (highlighted) were found in the same linkage group (LG032). HK7 was also found by us in one of the chromosome sequences reported 3 , but other HK loci (including HK26) were not found in the reported chromosome sequences, presumably because the sequences are not complete, or because the SSR loci are null in the sequenced genome. Reference details are provided in footnote

SSR primer pair ID [z]	SSR primer target sequence		Major linkage group (LG) [y]	cv. Jiangsu chromosome [x]	
	Genebank ID [z]	length (bp)		Genbank ID[x]	1st base position for primer target
HK5	EU532198.1	246	-	CM024753.1	45,720,051
HK7	EU532199.1	228	LG03	CM024760.1	67,924,214
HK22	EU532200.1	256	-	CM024757.1	165,875,866
HK25	EU532201.1	159	-	CM024759.1	75,888,016
HK26	EU532202.1	210	LG03	-	-
HK34	EU581819.1	304	-	CM024753.1	156,550,046
HK35	EU581820.1	304	minor linkage group	-	-

[z] HU K., HUANG X. F., KE D., DING Y. I., 2009 - *Characterization of 11 new microsatellite loci in taro (*Colocasia esculenta*).* - Mol. Ecol. Res., 9: 582-584.

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Adv. Hort. Sci., 2021 35(3): 269-275 DOI: 10.36253/ahsc-10193

AHS
Advances in Horticultural Science

Effect of vine and fruit pruning on yield attributes of two watermelon (*Citrullus lanatus*) cultivars

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Key words: Apical shoot pinching, cucurbits, fruit pinching, fruit size, hybrid.

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Citation:
NDEREYIMANA A., WAWERU B.W., KAGIRANEZA B., NIYOKURI A.N., RUKUNDO P., HAGENIMANA G., 2021 - *Effect of vine and fruit pruning on yield attributes of two watermelon (Citrullus lanatus) cultivars.* - Adv. Hort. Sci., 35(3): 269-275

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 27 December 2020
Accepted for publication 16 July 2021

Abstract: This study was carried out to determine the effect of vine and fruit pruning on watermelon (*Citrullus lanatus*) yield. Five pruning methods: P1=no pruning at all, P2=pruning to four vines with two fruits per vine, P3=pruning to four vines with one fruit per vine, P4=pruning to three vines with two fruits per vine and P5=pruning to three vines with one fruit per vine were evaluated on two watermelon cultivars: 'Sugar baby' and 'Julie F1' under a factorial randomized complete block design with three replications. Investigations were carried out in the seasons 2017A (short rains) and 2017B (long rains) at Karama and Rubona experimental sites belonging to Rwanda Agriculture and Animal Resources Development Board. The obtained results indicated a significant difference among the different cultivars and pruning methods tested during both seasons and at two sites. Generally, all studied parameters recorded higher values during season 2017B than in season 2017A at Rubona site. A similar trend was recorded at Karama site except that the fruit yield per plant and per hectare for plants which were pruned to three vines with one fruit reduced during season 2017B as compared to season 2017A. The highest number of fruits per plant, fruit weight, fruit yield per plant and per hectare was recorded in 'Julie F1' compared to 'Sugar baby' at both sites and during both seasons. Higher fruit weight was obtained when both cultivars were pruned to three or four vines with one fruit per vine. Higher number of fruits per plant and higher fruit yield per plant was observed under pruning to four vines with two fruits per vine at Rubona site; while at Karama site, higher fruit yield per plant was recorded under pruning to three vines with one fruit or two fruits per vines and pruning to four vines with two fruits per vine. A similar trend was observed in fruit yield per hectare. Based on results of the current study, cultivation of the hybrid 'Julie F1' and pruning to three vines with one fruit per vine is recommended for optimum watermelon yield with big-sized fruits.

1. Introduction

Watermelon is a crop belonging to the Cucurbitaceae family that has gained a great economic importance due to its delicious fruits that are also rich in various nutritional compounds (Kong *et al*., 2017). During the

year 2018, the worldwide watermelon production was 103,931,337 t harvested over an area of 3,241,239 ha with the yield of 32.1 t ha^{-1} (FAO, 2020); while in East Africa, it was 230,729 t from an area of 12,110 ha with the yield of only 19.1 t ha^{-1}. The yield gap of 13 t ha^{-1} compared to the world's yield can be addressed by improving production practices, including regulation of number of vines and fruits per plant (Oga and Umekwe, 2016; Dube *et al*., 2020).

As other cucurbits, watermelon is also a crop with vines on which female flowers appear after about every five male flowers (Dube *et al*., 2020). The number of vines per plant is an important parameter determining the performance of Cucurbitaceae crops, including watermelon (Gomes *et al*., 2019). On the other side, the number of fruits per vine is also an important parameter that determines fruit size, mass and yield (Lins *et al*., 2013). Therefore, due consideration given to these aspects of cucurbit management is of utmost importance. In cucurbits, regulation of number of vines per plant and fruits per vine can be achieved through different methods including vine and fruit pruning (Campos *et al*., 2019).

Pruning is a special horticultural practice that is carried out by removing some parts of plant to boost flowering and subsequent fruiting. This leads to improved yield with enhancement in features required by consumers, such as fruit size and appearance (Oga and Umekwe, 2016). The main purpose of pruning is to promote balance between vegetative growth and fruit load (Anwar *et al*., 2019). Pruning has been reported to increase yield of cucumber (Nayak *et al*., 2018) and butternut (Eve *et al*., 2016). Pruning is also a common practice in watermelon production that can lead to synchronization of harvesting period and production of uniform fruits (Oga and Umekwe, 2016).

In East African countries, limited investigations have been carried out on watermelon as compared to legume and cereal crops (Dube *et al*., 2020). Besides, East African farmers rarely practice watermelon pruning due to limited knowledge on appropriate pruning method. The study was carried out to determine the effect of vine and fruit pruning on yield attributes of two watermelon cultivars.

2. Materials and Methods

Sites and seasons of study

Field experiment was carried out at Karama and Rubona experimental sites belonging to Rwanda Agriculture and Animal Resources Development Board (RAB). Karama site is located in Eastern Province, Bugesera District, on longitude 02°23'15"S, latitude 30°11'27"E, and at an altitude of 1524 m above sea level (Ndabamenye *et al*., 2013; Kabirigi *et al*., 2017). Its annual average rainfall is 850-1100 mm and the average temperature is 20-21°C (Verdoodt and Van Ranst, 2003). Rubona site is located in Southern Province, Huye District, on longitude 029°46'475"E, latitude 02°29'327"S, and at an altitude of 1727 m above sea level (Ndabamenye *et al*., 2013; Kabirigi *et al*., 2017). The annual average rainfall at Rubona is 1100-1400 mm and the average temperature is 17-20°C (Verdoodt and Van Ranst, 2003).

At both sites, the study was conducted in two cropping seasons: Season 2017A (short rain season) covering the period from September to December 2016 and Season 2017B (long rain season) from February to May 2017.

Study design and treatments

The study was conducted in a factorial randomized complete blocks design (RCBD) where treatments were replicated thrice. The treatment structure consisted of five different pruning methods (factor one) evaluated on two watermelon cultivars (factor two). The five pruning methods were designed as follows: P1=No pruning at all (control), P2=Pruning to four vines with two fruits per vine, P3=Pruning to four vines with one fruit per vine, P4=Pruning to three vines with two fruits per vine, and P5=Pruning to three vines with one fruit per vine. The two studied watermelon cultivars were C1: 'Sugar baby' and C2: 'Julie F1'. These cultivars were selected because of their high yield potential under breeder's conditions. Moreover, 'Julie F1' is a popular hybrid among farmers in East African Region. In total, ten treatment combinations (cultivar x pruning method) were studied.

Trial establishment and maintenance

Field was ploughed twice at an interval of two weeks and was subdivided into plots of 6 m x 3 m that were mulched using dry grass. There were ten plots in each replication; and each plot received a treatment combination of cultivar and pruning method. Watermelon seedlings for each cultivar were raised in biodegradable pots and transplanted 30 days after sowing at a spacing of 3 m x 2 m. Pruning was achieved by pinching the apical shoot of watermelon vine after six nodes, about 18 days after

transplanting (Anwar *et al*., 2019). Then, at 15 days after pinching, four vines were maintained for P2 and P3 treatments while three vines were maintained for P4 and P5. Thereafter, two fruits were maintained per vine for P2 and P4 while one fruit was maintained per vine for P3 and P5. For all the treatments where pruning was carried out, one of the maintained vines was not allowed to have fruits for it to support other in feeding their fruits.

Apart from watermelon cultivars and pruning methods, other crop management practices such as fertilization, watering, weeding, pests and diseases management were carried out as recommended. Fertilization was conducted by applying organic manure and the inorganic fertilizer N:P:K at the rates of 25 t ha^{-1} and 90:60:60 kg ha^{-1}, respectively. The inorganic fertilizer NPK 17-17-17 was used to supply 60:60:60 kg ha^{-1}, which was given in two equal splits (at transplanting and at one month after transplanting); while additional 30 kg of N ha^{-1} was supplied in form of urea 46% and was given at two months after transplanting. Where necessary, copper oxychloride and carbendazim pesticides were sprayed to control diseases while Lambda-Cyhalothrin and imidacloprid were used against pests.

Data collection and analysis

Observations were carried out on four randomly selected plants per each treatment combination. Data were recorded on number of fruits per plant, fruit weight, fruit yield per plant and fruit yield per hectare. The number of fruits per plant was obtained at harvesting through counting all available fruits on four selected plants per treatment combination followed by calculating the average per plant. Fruit weight (kg) was achieved as the average of five fruits randomly selected from the fruits harvested on the four pre-selected observational plants. Fruit yield per plant (kg) was recorded as the average weight of all fruits harvested from four observational plants in the plot under consideration. Fruit yield per hectare was derived from computation using the data on fruit yield per plant. Analysis of variance for the collected data was performed using GenStat 14th Edition software package and the level of significance was set at $P<0.05$. Least significant difference (LSD) test was conducted for pair-wise comparisons of means.

3. Results

The number of fruits per plant differed significantly ($P<0.05$) between cultivars in both seasons (2017A and 2017B) at Karama site. Significant differences among pruning methods were recorded only in season 2017A at Karama and in both seasons at Rubona. Between the two cultivars, the highest number of fruit per plant was recorded in 'Julie F1' (3.5-4.0 fruits per plant) compared to 'Sugar baby' (3.4-3.7 fruits per plant). For pruning method, the highest number of fruit per plant (4.1-4.8 fruits per plant) at Karama site was recorded when both watermelon cultivars were pruned to four vines with two fruits per vine (Table 1). A similar trend was also observed at Rubona where both watermelon cultivars were pruned to four vines with two fruits per vine recorded the highest number of fruits per plant (4.8-5.0) (Table 2). There was no significant interaction among cultivars and pruning methods on the number of fruits per plant.

Fruit weight differed significantly ($P<0.05$) between cultivars and among pruning methods. The highest fruit weight was recorded in plots where 'Julie F1' was planted at both sites and it varied between 3.8-3.9 kg per fruit at Karama site (Table 1) and 3.4-3.8 kg per fruit at Rubona site (Table 2). Among pruning methods, the highest fruit weight (4.2-4.3 kg at Karama and 3.7-4.1 kg at Rubona) was obtained when both cultivars were pruned to three vines with one fruit per vine during season 2017A and 2017B (Table 1 and 2). The interaction of cultivars and pruning methods on fruit weight in both 2017A and 2017B seasons at Rubona and at Karama during the season 2017A was significantly different. However, it was not significantly different in the season 2017B at Karama site. Higher fruit weight (4.1 4.6 kg per fruit) was obtained under 'Julie F1' pruned to three vines with one fruit per vine, followed by the same cultivar pruned to four vines with one fruit per vine (3.7-4.2 kg per fruit) (Tables 1 and 2).

Fruit yield per plant and per hectare was significantly ($P<0.05$) different between cultivars and among pruning methods and their interaction in both seasons 2017A and 2017B, and at Karama and Rubona sites was not relevant. 'Julie F1' recorded significantly higher fruit yield per plant (11.9-15.4 kg) and per hectare (59.6t ha^{-1} -76.9t ha^{-1}) compared to Sugar baby which recorded 9.5-11.2 kg per plant and 47.5t-59.1t per hectare. Among the pruning methods, the highest fruit yield per plant (13.9-15.9 kg) and yield per hectare (69.3 t ha^{-1} -78.0 t ha^{-1}) was observed under pruning to four vines with two fruits per vine at Rubona site (Table 2). At Karama site,

Table 1 - Performance of watermelon as affected by cultivars and pruning method, Karama Site

Treatments	No. of fruits per plant		Fruit weight (kg)		Fruit yield (kg per plant)		Fruit yield (t ha^{-1})	
	Season 2017A	Season 2017B	Season 2017A	Season 2017B	Season 2017A	Season 2017B	Season 2017A	Season 2017B
Factor 1: Cultivar (C)								
C1	3.4 b	3.7 b	3.2 b	3.2 b	10.8 b	11.2 b	54.1 b	59.1 b
C2	3.8 a	4.0 a	3.8 a	3.9 a	14.8 a	15.4 a	74.0 a	76.9 a
Significance	***	***	***	***	***	***	***	***
SED	0.095	0.159	0.060	0.063	0.439	0.644	2.19	3.22
Factor 2: Pruning method (P)								
P1	3.0 c	3.4	2.8 d	2.9 e	8.6 c	10.0 c	43.2 c	49.7 c
P2	4.1 a	4.8	3.2 c	3.3 d	13.5 b	15.8 a	67.3 b	78.9 a
P3	3.5 b	3.4	3.8 b	3.8 b	13.4 b	13.2 b	67.0 b	65.7 b
P4	3.6 b	4.0	3.5 c	3.5 c	12.8 b	14.2 ab	63.8 b	71.1 ab
P5	3.7 ab	3.5	4.2 a	4.3 a	15.8 a	14.9 ab	78.8 a	74.5 ab
Significance	***	NS	***	***	***	***	***	***
SED	0.151	0.251	0.094	0.099	0.694	1.019	3.47	5.09
Interaction (C x P)								
C1P1	2.8	3.1	2.7 f	2.7	7.7	8.5	38.3	42.4
C1P2	4.0	4.7	3.6 cd	2.9	11.5	13.7	57.4	68.3
C1P3	3.3	3.4	3.4 de	3.4	11.1	11.7	55.5	58.6
C1P4	3.3	3.8	3.1 ef	3.1	10.3	11.9	51.5	59.7
C1P5	3.6	3.4	3.8 bcd	3.9	13.5	13.3	67.7	66.6
C2P1	3.3	3.8	2.9 ef	3.0	9.6	11.4	48.0	57.0
C2P2	4.3	4.9	2.9 ef	3.6	15.4	17.9	77.2	89.5
C2P3	3.7	3.4	4.2 ab	4.2	15.7	14.6	78.5	72.9
C2P4	3.9	4.2	3.9 bc	3.9	15.2	16.5	76.2	82.6
C2P5	3.9	3.6	4.6 a	4.6	18.0	16.5	90.0	82.4
Significance	NS	NS	***	NS	NS	NS	NS	NS
SED	0.213	0.355	0.133	0.140	0.981	1.441	4.91	7.20

C1=Sugar baby, C2=Julie F1, P1=No pruning, P2= Pruning to 4 vines with 2 fruits per vine, P3= Pruning to 4 vines with 1 fruit per vine, P4= Pruning to 3 vines with 2 fruits per vine, P5= Pruning to 3 vines with 1 fruit per vine, 2017A=short rains, 2017B=long rains.
SED= Standard error difference.
* and ** =significant difference at 5% and 1% respectively, NS=not significantly different (P>0.05).
Means within each column followed by the same letter are not significantly different as per LSD test at P<0.05.

higher fruit yield per plant (15.8 kg) and per hectare (78.8 t ha^{-1}) was recorded under pruning to three vines with one fruit per vine during season 2017 A (Tables 1) while during season 2017B, higher fruit yield per plant and per hectare was recorded on watermelon plants that were pruned to three vines with one fruit per vine or four vines with one fruit or two fruits per vine (14.2-15.8 kg per plant and 71.1 t ha^{-1} -78.9 t ha^{-1}) (Table 1).

4. Discussion and Conclusions

Among others, crops of *Cucurbitaceous* family can be manipulated by altering the source: sink ratio through stem and fruit pruning, which affects the number of leaves per plant and consequently leaf area (source) and demand for photoassimilates (sink) (Queiroga *et al*., 2008). In this family, more pruning studies have been carried out on other crops such as cucumber (Nayak *et al*., 2018) and butternut (Eve *et al*., 2016); there is limited information on this aspect as far as watermelon is concerned. Therefore, the current study contributed valuable knowledge on watermelon management in a view to optimize its production.

Heavier fruits obtained under watermelon plants (Julie F1) pruned to three or four vines with one fruit per vine fall in line with Lins *et al*. (2013) who reported that fruit thinning allows larger amounts of produced photo-assimilates to be used by few fruits causing them to attain a size demanded on market. Similar to results of the current study, Dhillon *et al*. (2017) also obtained heavier fruits on pruned cucum-

Table 2 - Performance of watermelon as affected by cultivar and pruning method, Rubona Site

Treatments	No. of fruits per plant		Fruit weight (kg)		Fruit yield (kg per plant)		Fruit yield (t ha^{-1})	
	Season 2017A	Season 2017B	Season 2017A	Season 2017B	Season 2017A	Season 2017B	Season 2017A	Season 2017B
Factor 1: Cultivar (C)								
C1	3.4	3.6	2.8 b	3.1 b	9.5 b	11.1 b	47.5b	54.3b
C2	3.5	3.8	3.4 a	3.8 a	11.9 a	14.2 a	59.6a	69.9a
Significance	NS	NS	***	***	***	***	***	***
SED	0.198	0.182	0.051	0.057	0.617	0.643	3.08	3.10
Factor 2: Pruning method (P)								
P1	3.0 b	3.2 b	2.5 e	2.8 e	7.6 c	9.0 c	38.0 c	44.0 c
P2	4.8 a	5.0 a	2.9 d	3.2 d	13.9 a	15.9 a	69.3 a	78.0 a
P3	3.0 b	3.2 b	3.4 b	3.7 b	10.1 b	12.1 b	50.5 b	59.0 b
P4	3.5 b	3.7 b	3.1 c	3.4 c	10.9 b	12.9 b	54.3 b	63.7 b
P5	3.0 b	3.2 b	3.7 a	4.1 a	11.2 b	13.3 b	55.8 b	65.9 b
Significance	***	***	***	***	***	***	***	***
SED	0.312	0.288	0.081	0.090	0.975	1.016	10.25	4.90
Interaction (C x P)								
C1P1	2.7	2.9	2.4 f	2.7 f	6.5	7.7	32.3	37.4
C1P2	5.0	5.0	2.6 ef	2.9 ef	12.9	14.3	64.3	69.9
C1P3	3.0	3.2	3.0 d	3.3 d	9.0	10.8	45.0	52.5
C1P4	3.3	3.6	2.7 e	3.0 e	9.1	10.8	45.5	52.9
C1P5	3.0	3.2	2.4 c	3.7 c	10.1	12.1	50.5	59.0
C2P1	3.3	3.6	2.6 ef	2.9 ef	8.7	10.4	43.7	50.6
C2P2	4.7	5.0	3.2 cd	3.6 cd	14.9	17.6	74.3	86.1
C2P3	3.	3.2	3.7 b	4.2 b	11.2	13.4	56.0	65.5
C2P4	3.7	3.9	3.4 c	3.8 c	12.6	14.9	63.2	74.5
C2P5	3.0	3.2	4.1 a	4.5 a	12.2	14.6	61.0	72.9
Significance	NS	NS	*	*	NS	NS	NS	NS
SED	0.442	0.407	0.115	0.127	1.379	1.437	6.90	6.93

C1=Sugar baby, C2=Julie F1, P1=No pruning, P2= Pruning to 4 vines with 2 fruits per vine, P3: Pruning to 4 vines with 1 fruit per vine, P4= Pruning to 3 vines with 2 fruits per vine, P5= Pruning to 3 vines with 1 fruit per vine, 2017A=short rains, 2017B=long rains.
SED= Standard error difference.
* and **=significant difference at 5% and 1% respectively, NS=not significantly different (P>0.05).
Means within each column followed by the same letter are not significantly different as per LSD test at P<0.05.

ber plants compared to unpruned ones. These findings could be further explained by the fact that plants with less branches allow more light interception and this leads to improved photosynthesis, more accumulation of carbohydrates, and thus overall improved crop performance as compared to plants with relatively higher number of branches (Feng *et al*., 2008). In agreement with the findings of current study, Ali *et al.* (2016) also reported interaction effect of cultivars and pruning on yield of bottle gourd (*Lagenaria siceraria*).

In this study, fruit and vine pruning have been observed to significantly affect watermelon yield. This would be attributed to the fact that stem and fruit density is among agronomic variables associated with yield performance of vegetable crops (Ayala-Tafoya *et al.*, 2019). The number of stem per plant contributes to plant density, which affects distribution and utilization of soil nutrients and solar energy (Rahmatian *et al*., 2014). According to Campos *et al*. (2019), efficient solar radiation and production of photo-assimilates are the important pre-requisites for optimum watermelon production. The number of vines per plant also affects the root volume and plant vigour, which in turn influences water and nutrients' uptake and availability. Viana *et al*. (2008) reported that the lower the number of vine per plant, the higher the root volume and plant vigour and finally the higher yield as a result of improved nutrient and water uptake. The lower yield recorded on watermelon plants without pruning could result from competition for water, nutrients and light (Gomes *et al*., 2019). Similarly, Muñoz-Rengifo *et al*. (2018) argued that since watermelon has naturally many branches,

pruning is advised to keep adequate number of branches, leaves and fruits to enable them to share efficiently the plant resources. In agreement with findings of the current study, Douglas *et al*. (2001) and Palada and Chang (2003) also reported a significant effect on yield of cucumber and bitter gourd through pruning by removal of lateral shoots.

Based on results of the current study, pruning to three vines with one fruit per vine is therefore, recommended for optimum yield with big-sized fruits of both watermelon cultivars ('Julie F1' and 'Sugar baby').

Acknowledgements

We thank the Government of Rwanda for supporting this study through the project "Development of market-responsive plant varieties and seed systems to reduce dependence on importation". Authors are thankful to Dative Niyomufasha, Innocent Ndutiye, Eric Sibomana, Immaculée Mukabanana, and Raurence Mukanziza for their enthusiastic involvement in field work.

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Adv. Hort. Sci., 2021 35(3): 277-284 DOI: 10.36253/ahsc-10523

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Advances in Horticultural Science

Genotypic diversity and trait profiles of some *Amaranthus* accessions

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Key words: *Amaranthus*, biplot, improvement, trait profile, variability.

Abstract: Knowledge of the pattern of trait variation among accessions, and the trait profiles of the accessions is crucial for improvement of a crop. Twenty-one *Amaranthus* accessions were evaluated in 2018 and 2019 to investigate the extent of genotypic diversity among the amaranth accessions and their trait profiles. Data were subjected to analysis of variance, and correlation and principal component analyses. Taking stem weight (SWT) as the yield, the accession × yield-trait combination (GYT) biplot was employed to investigate the trait profiles of the accessions. Accession, year, and accession × year mean squares were significant (P≤0.05/0.01) for most of the measured traits. The first three principal components explained 88.18% of observable variation among the accessions and identified plant height (PHT), number of leaves per plant (NOL), and root weight (RWT) as the major contributors. Significant (P≤0.01) correlation was observed in the association of SWT with NOL, TBM, and RWT. Accessions NGB00019 and NGB00061 were associated with the sector containing all the YT combinations considered. However, NGB00019 was identified as the best combiner of yield with other traits. Further studies involving more traits should determine the trait profiles of the remaining accessions.

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Citation:
OYETUNDE O.A., OLAYIWOLA M.O., OSHO B.T., 2021 - *Effect of vine and fruit pruning on yield attributes of two watermelon Citrullus lanatus) cultivars.* - Adv. Hort. Sci., 35(3): 277-284

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 26 February 2021
Accepted for publication 2 August 2021

1. Introduction

Amaranthus is a member of the Amaranthaceae family and is believed to have originated from South America (Janovská *et al*., 2012). Despite the level of under-development, the genus is one of the most diverse among cultivated crops, with about 70 species (Ebert *et al*., 2011). Amaranths are classified based on the part of the plant for which they are grown. The leaf types including *A. hybridus* and *A. tricolor* are grown for their leaves consumed as vegetables while the grain amaranths such as *A. caudatus*, *A. cruentus*, and *A. hypochondriacus* are popular for the grains. In addition, some amaranth like *A. tricolor* are valued as ornamental, while some (e.g. *A. palmeri* S. Wats., *A. powellii S. Watt., A. retroflexus L.*, and *A.*

spinosus L.) are considered as weeds. In general, cultivated amaranths are of nutritional importance because they produce edible leaves, stems, and grains (e.g., A. *hybridus* and A. *tricolor*) (Akin-Idowu *et al*., 2016; Neelesh and Pratibha, 2018). The leaves and stem are rich natural stores of vitamins (A, B, and C), and dietary minerals including calcium and iron (Stallknecht and Schulz-Schaeffer, 1993). *Amaranthus* has also been found to be a source of lysine, an essential amino acid that is lacking in diets based on cereals and tubers (Schippers, 2000). The vegetable is reported to be adapted to a wide range of agro-ecology habitat (Katiyar *et al*., 2000) and can tolerate biotic (i.e. diseases and pests) and abiotic (i.e. heat and drought) stresses (Shukla *et al*., 2010). As is with many other under-utilized leaf vegetables, the genetic and economic potentials of *Amaranthus* have not been fully harnessed.

Although the amaranth is essentially self-pollinating, there is significant level of natural outcrossing and inter-specific and inter-varietal hybridization, leading to the occurrence of wide differences among accessions (Akin-Idowu, 2016). There is a growing interest in research on amaranth because of the high genetic diversity and phenotypic variability of the crop which are of immense use in designing strategies for improvement of the crop. Plant breeders have found differences in plant observations among genetic materials as effective means to deduce estimates of genetic diversity (Akin-Idowu, 2016) and to determine genotypic superiority. However, identification of genetic superiority is hindered by unfavourable associations among a set of target traits since the decision is often based on multiple traits (Yan and Fregeau-Reid, 2018). This implies that a successful cultivar must attain desirable levels for several key traits. Thus, knowledge of the pattern of trait variation among accessions and the trait profile (strength and weakness) of available Amaranth accessions will aid the exploitation of existing genotypic diversity for improvement of the crop for desired traits. Recently, the accession x yield-trait (GYT) biplot approach was proposed by Yan and Fregeau-Reid (2018) to combat the challenge posed by evaluation of accessions based on multiple traits, In this approach, the worth of a accession is determined by its value for yield in combination with other traits (Y-T) rather than its levels for an individual trait. Therefore, this study investigated the extent of genotypic diversity among amaranth accessions, as well as the trait profiles of the accessions with a view to providing information to aid effective future improvement strategies.

2. Materials and Methods

Genetic materials

Twenty-one (21) amaranthus accessions (NGB00001, NGB00005, NGB00019, NGB00022, NGB00025, NGB00027, NGB00028, NGB00029, NGB00031, NGB00024, NGB00058, NGB00059, NGB00060, NGB00061, NGB00070, NGB00078, NGB00082, NGB00108, NGB00111, NGB00112, and a local check named LASPO-COL-001). All the accessions, except the local check, were obtained from the National Agency for Crop Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria. Seeds of the local check were collected from a reputable farmer in Ikorodu. All the accessions belong to the species *A*. *hybridus*, and are of Nigerian origin (Table 1).

Field evaluation and phenotyping

The 21 amaranthus accessions were evaluated on the field during the main seasons of 2018 and 2019.

Table 1 - Origin of the 21 Amaranthus hybridus accessions used in the study

Serial number	Accession name	Origin
1	NGB00001	Katsina State, Nigeria
2	NGB00005	Kebbi State, Nigeria
3	NGB00019	Lagis State, Nigeria
4	NGB00022	Zamfara State, Nigeria
5	NGB00025	Niger State Nigeria
6	NGB00027	Osun State, Nigeria
7	NGB00028	Osun State, Nigeria
8	NGB00029	Ondo State, Nigeria
9	NGB00031	Oyo State, Niigeria
10	NGB00034	Oyo State, Niigeria
11	NGB00058	Ogun State, Nigeria
12	NGB00059	Ondo State, Nigeria
13	NGB00060	Oyo State, Niigeria
14	NGB00061	Osun State, Nigeria
15	NGB00070	Ogun State, Nigeria
16	NGB00078	Oyo State, Niigeria
17	NGB00082	Oyo State, Niigeria
18	NGB00108	Oyo State, Niigeria
19	NGB00111	Oyo State, Niigeria
20	NGB00112	Oyo State, Niigeria
21	LASPO-COL-001	Lagis State, Nigeria

The land was initially tilled mechanically by ploughing twice and then harrowing. Subsequently, raised beds; 2×1 m in diameter, were made manually. Cured poultry manure was applied on the bed at a rate of 10 tons/ha. The trial was laid out in randomized Complete Block Design with two replications. Each accession was grown in a 2-row plot fitted into a bed in each replicate. A seed rate of 1.5 kg/ha was used, and planted by drilling. Seeds were mixed with dry fine sand to enhance even distribution within the drills. The experiment was exclusively rain-fed. Weeds were controlled manually by rogueing, subject to field inspection, and chemical insecticides were not used throughout the experiment.

At maturity, a random sample of five plants per row; making 10 plants per replicate per accession, were observed for plant height (PHT) (cm), number of leaves per plant (NOL), and stem girth (STG) (mm). All the plants in each plot were uprooted, and the roots washed carefully, to record data on total biomass (TBM) (g), root weight (RWT) (g), stem weight (SWT), and harvest index (%). Observations were recorded according to the Amaranthus descriptors of IPGRI (1999). Data on TBM, RWT, and SWT were converted to kg/ha. Harvest index was computed as the percent of the total biomass that is made up by the stem weight.

Data analyses

Test for homogeneity of variance was not significant and thus analysis of variance (ANOVA) was performed on combined data from the two-year evaluation using 'proc glm' in SAS (SAS, 2011). Means of data collected for each accession were subjected to correlation (Pearson coefficients) analysis among all pair-wise combinations of measured traits while the standardized mean values were subjected to principal component analysis. To reveal the level of phenotypic divergence among pairs of accessions, estimates of genetic distance (Euclidean) among all possible pairs of accessions were obtained using 'proc distance' while the accessions were distributed into clusters from dendrogram obtained using 'proc tree' (SAS, 2011). To investigate the trait profiles of the accessions, a accession × yield-trait combination (GYT) (Yan and Fregeau-Reid, 2018) biplot approach was employed where stem weight was taken as the yield. The GYT biplots were obtained using the GGEBiplotGUI package in R. To select accessions for the GYT biplots, a superiority index (SI) was computed for the accessions, based on standardized GYT estimates. The SI value of a accession was computed as the arithmetic mean of its standardized estimates. Ten accessions comprising seven and three accessions with the highest and lowest SI values respectively were selected for the GYT biplot.

3. Results

Results of analysis of variance of amaranth accessions are summarized in Table 2. Mean squares of accession were significant (P≤0.05/0.01) for all the measured traits except stem girth, while mean squares of year were significant (P≤0.05/0.01) for number of leaves per plant, root biomass, and harvest index. Accession × year mean squares were also found to be significant (P≤0.05/0.01) for plant height, number of leaves per plant, and harvest index.

The first three principal components (PCs) jointly accounted for 88.18% of the total variation among accessions, with PCs 1, 2, and 3 having Eigen values of 2.64, 1.39, and 1.25, respectively, and explaining 44.06, 23.21, and 20.91% of the total variation in that order (Table 3). Plant height, root weight, and number of leaves per plant had high loadings (≥0.30) of

Table 2 - Mean squares of measured traits of *Amaranthus* evaluated in 2018 and 2019

Source of variation	DF	PHT	NOL	STG	TBM	RWT	SWT	HI
Rep (Year)	2	4.160	7.043**	0.010	0.039	0.005	0.023	0.002
Year	1	0.481	7.346*	0.004	0.007	0.036**	0.061	0.264**
Accession	20	10.989**	9.745**	0.014	0.057**	0.007**	0.031*	0.015**
Accession × Year	20	5.800**	6.517**	0.011	0.019	0.004	0.011	0.022**
Error	40	2.110	0.332	0.008	0.023	0.049	0.015	0.005

Rep = Replicate; DF = degrees of freedom; PHT = Plant height; NOL = Number of leaves per plant; STG = Stem girth; TBM = Total biomass; RWT = Root weight; SWT = Stem weight; HI = Harvest index.
* and ** significant at 5 and 1% probabilities respectively.

Table 3 - Loadings from principal component axes of the compost used in this study

Trait	Principal component axis 1	Principal component axis 2	Principal component axis 3
Plant height	0.55	-0.14	0.09
Number of leaves per plant	0.44	0.21	0.40
Stem girth	0.25	-0.14	0.57
Root weight	0.50	0.02	-0.49
Stem weight	0.27	0.66	-0.35
Harvest index	-0.23	0.69	0.37
Eigen value	2.64	1.39	1.25
Proportion (%) of variation	44.06	23.21	20.91
Cumulative (%) variation	44.06	67.27	88.18

0.55, 0.50, and 0.44 respectively in PC 1. High loadings in PC 2 were 0.69 and -0.66 observed for harvest index and stem weight respectively while PC 3 was characterized by stem girth, root weight, number of leaves per plant, harvest index, and stem weight with loadings of 0.57, -0.49, 0.40, 0.37, and -0.35, respectively. The PCA biplot grouped the amaranthus accessions into clusters over the four quadrants based on the contributions of the measured traits as explained by the PCs (Figs. 1 and 2). The accessions were scattered on the score biplot with the local check distinctly placed. Accessions NGB00022, NGB00031, NGB00060, and NGB00070 in the top left quadrant were associated with harvest index. The top right quadrant, associated with number of leaves per plant and stem and root weights, had NGB00005, NGB00019, NGB00025, NGB00058, NGB00061, and

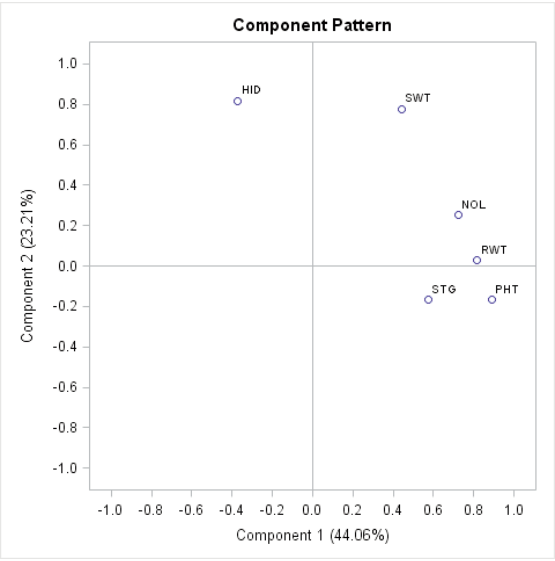

Fig. 1 - Principal component loading pattern of six traits of *Amaranthus* accessions.

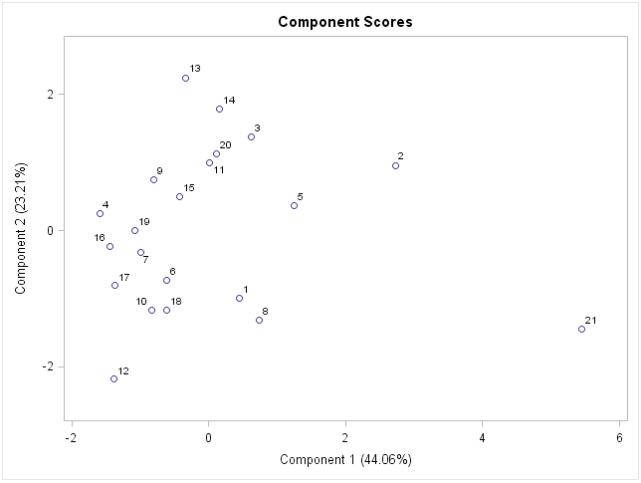

Fig. 2 - Principal components score plot for 21 Amaranthus accessions.

NGB00112 as the corresponding accessions. The bottom right quadrant, characterized by plant height and stem girth, was composed by NGB00001, NGB00029, and the Local check. Other accessions, clustered in the bottom left quadrant, were not associated with any of the traits measured in this study.

Estimates of correlation coefficient revealed significant (P≤0.05/0.01) associations among pairs of measured traits (Table 4). Positive and significant (P≤0.05/0.01) correlation was observed in the association of plant height with number of leaves per plant (r= 0.52), stem girth (r= 0.27), total biomass (r= 0.29), and root weight (r= 0.47). Number of leaves per plant showed positive and significant (P≤0.05) correlation with stem girth, total biomass, root weight and stem weight with a correlation coefficient of 0.27, 0.24, 0.22 and 0.22, respectively. Furthermore, total biomass and root weight had a significant positive correlation (r= 0.70; P≤0.01) while there was positive and significant correlation in the association of stem weight with total biomass, root weight and harvest index (r= 0.93, 0.43, and 0.27, respectively). Finally, harvest index had negative and significant correla-

Table 4 - Pearson correlation coefficients among measured traits of 21 *Amaranthus* accessions evaluated in 2018 and 2019

	NOL	STG	TBM	RWT	SWT	HI
PHT	0.52**	0.27*	0.29**	0.47**	0.14	-0.27*
NOL		0.27*	0.24*	0.22*	0.22*	0.10
STG			0.16	0.14	0.15	-0.02
TBM				0.70**	0.93**	-0.04
RWT					0.43**	-0.68**
SWT						0.27*

Re* and ** significant at 5 and 1% probabilities respectively.

tions (P≤0.05/0.01) with plant height (r= -0.27) and root weight (r= -0.68).

The estimates of genetic distance among pair-wise combination of accessions based on composited traits (Table 5) ranged from 0.99 between NGB00028 and NGB00111 to 7.90 between NGB00078 and the local check. In particular, high genetic distance estimates were obtained between the local check and NGB00022 (7.45), NGB00059 (7.72), NGB00060 (7.02), and NGB00082 (7.21).

At a genetic dissimilarity of 0.50, the grouping of the accessions by the dendrogram (Fig. 3) fairly compares with the groupings on the PC scores biplot. For instance, cluster 1 was composed of eight accessions, six of which were NGB00060, NGB00058, NGB00112, NGB00025, NGB00061, and NGB00019 which were grouped together, and associated with total biomass and stem and root weights on the PC scores biplot. The other two accessions in cluster I, NGB00031 and NGB00070, were also grouped together on the PC score plot, and were associated with harvest index. Cluster 2 contained NGB00005 and the local check. The two accessions were also grouped together by the PC score biplot, and associated with plant height, number of leaves per plant, and stem girth. Similarly, all the accessions in cluster 3; NGB00001, NGB00029,

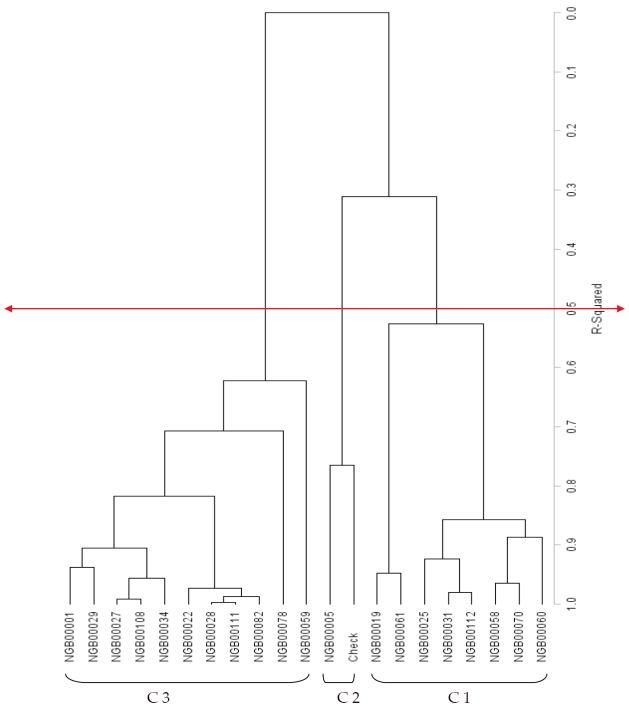

Fig. 3 - Dendrogram of relatedness among *Amaranthus* accessions, (X-axis) based on genetic dissimilarity (Y-axis) from Single-Linkage Cluster analysis. The red double-arrowed line delineates the accessions into clusters at approximately 0.50 level of dissimilarity; C1, C2, C3 are clusters 1, 2, and 3, respectively.

Table 5 - Pair-wise genetic distance estimates based on observed phenotypes of 21 *Amaranthus* accessions

ID	ACCESSION	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	NGB00001	0.00	3.62	3.86	2.52	1.82	1.37	2.13	1.65	2.34	1.62	2.73	3.81	3.91	4.02	2.27	4.23	2.58	1.84	2.47	2.44	5.33
2	NGB00005		0.00	3.68	4.89	2.26	4.36	4.50	3.88	3.80	4.80	3.39	6.27	3.94	3.50	3.50	5.66	5.32	4.77	4.73	3.11	3.81
3	NGB00019			0.00	4.14	2.97	4.24	4.54	4.93	2.82	4.71	4.00	5.09	4.45	1.54	3.70	6.57	5.08	5.21	4.83	2.61	6.33
4	NGB00022				0.00	3.19	1.42	1.19	2.97	1.69	2.14	2.23	3.79	3.01	3.88	1.68	3.28	1.54	2.06	1.26	2.26	7.45
5	NGB00025					0.00	2.67	3.07	2.70	2.15	3.06	2.35	5.10	3.10	3.20	2.45	4.98	3.75	3.19	3.17	1.63	4.79
6	NGB00027						0.00	1.00	1.80	2.12	1.35	2.37	3.38	3.58	4.14	1.70	3.26	1.41	1.15	1.45	2.46	6.39
7	NGB00028							0.00	2.10	2.21	1.85	2.01	3.55	3.17	4.17	1.31	2.47	1.12	1.25	0.99	2.52	6.80
8	NGB00029								0.00	3.34	2.52	2.55	4.16	3.96	4.94	2.52	3.54	2.28	1.56	2.27	3.06	5.07
9	NGB00031									0.00	2.53	2.18	4.31	2.69	2.57	1.63	4.26	2.93	2.94	2.41	1.33	6.76
10	NGB00034										0.00	3.32	3.54	4.27	4.56	2.46	3.67	2.31	1.52	2.35	3.25	6.79
11	NGB00058											0.00	5.06	1.54	3.69	1.51	3.41	2.62	2.65	1.75	1.48	6.10
12	NGB00059												0.00	6.32	4.96	4.01	4.94	3.43	3.87	4.34	4.80	7.72
13	NGB00060													0.00	4.03	2.58	4.15	3.78	3.81	2.72	2.15	7.02
14	NGB00061														0.00	3.10	5.78	4.93	5.05	4.60	2.61	6.74
154.	NGB00070															0.00	2.90	2.32	2.29	1.81	1.78	6.36
16	NGB00078																0.00	2.88	2.87	2.73	4.50	7.90
17	NGB00082																	0.00	1.40	1.18	3.12	7.21
18	NGB00108																		0.00	1.44	3.18	6.50
19	NGB00111																			0.00	2.48	6.99
20	NGB00112																				0.00	6.00
21	Local check																					0.00

NGB00027, NGB00108, NGB00034, NGB00022, NGB00028, NGB00111, NGB00082, NGB00078, and NGB00059, except NGB00022 and NGB00001 were also grouped together on the PC scores biplot, and were not associated with any of the traits measured in this study.

The trait profiles of the *Amaranthus* accessions are displayed on the polygon view of the GYT biplot (Fig. 4). The polygon view revealed four sectors with the NGB00019, local check, NGB00060, and NGB00078 as the vertex accessions in their respective sectors. Accessions NGB00019 and NGB00061 belonged to sector 1 which was characterized by all the measured yield-trait (Y-T) combinations. This sector contained the highest ranked accessions. Sector 2 with the local check as vertex accession, also contained NGB00005 and NGB00025, thus comprising accessions that were next in rank to the accessions in sector 1. Similarly, the accessions in sector 3; NGB00060 and NGB00112, ranked next to those in sector 2 while the-poorest ranked accessions; NGB00082, NGB00059, and NGB00078, constituted sector 4. Sectors 2, 3, and 4 were not associated with any of the Y-T combinations considered in this study.

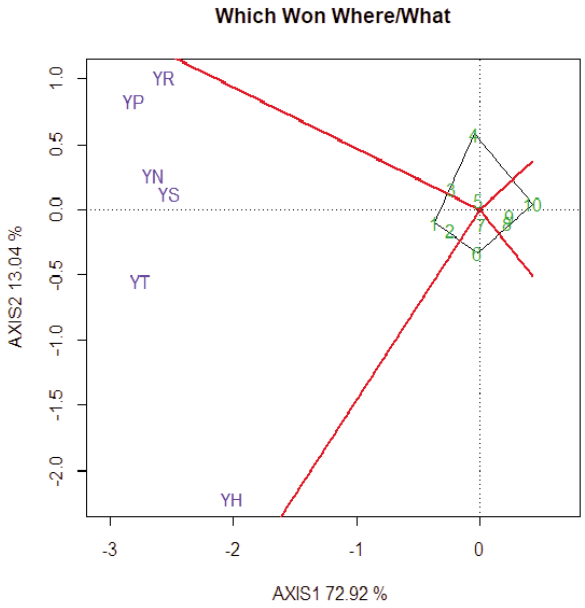

Fig. 4 - The accession × yield-trait biplot of 'which won where' of selected seven best and 3 worst *Amaranthus* accessions.

The names of the accessions 1 to 10 are available in Table 6.

Table 6 - Genotype × yield-trait combination data matrix for 21 *Amaranthus* accessions evaluated in 2018 and 2019

ID on GYT biplot	Accession	YxPHT	YxNOL	YxSTG	YxTBM	YxRWT	YxHI	Superiority index
1	NGB00019	1.779	1.183	1.386	2.337	2.299	1.774	1.793
2	NGB00061	1.291	1.564	0.793	1.903	1.701	1.655	1.485
3	NGB00005	1.426	1.923	1.337	1.200	1.470	0.943	1.383
4	Local check	1.564	1.081	1.098	0.553	1.476	-0.230	0.924
5	NGB00025	0.791	0.833	1.272	0.783	0.856	0.683	0.870
6	NGB00060	0.831	1.186	1.313	0.595	-0.367	1.576	0.856
7	NGB00112	0.886	0.676	1.005	0.766	0.462	1.087	0.814
Not selected	NGB00031	0.070	0.244	0.441	0.561	0.156	0.576	0.341
Not selected	NGB00058	0.427	0.394	0.423	0.005	-0.326	0.560	0.247
Not selected	NGB00070	-0.188	0.060	-0.251	-0.244	-0.245	0.017	-0.142
Not selected	NGB00001	-0.294	-0.365	-0.041	-0.295	0.003	-0.453	-0.241
Not selected	NGB00022	-0.499	-0.537	-0.340	-0.438	-0.531	-0.101	-0.408
Not selected	NGB00111	-0.584	-0.635	-0.466	-0.710	-0.826	-0.388	-0.602
Not selected	NGB00028	-0.685	-0.633	-0.707	-0.726	-0.740	-0.576	-0.678
Not selected	NGB00027	-0.758	-0.802	-0.692	-0.694	-0.490	-0.707	-0.690
Not selected	NGB00034	-0.978	-0.748	-0.593	-0.682	-0.499	-0.822	-0.720
Not selected	NGB00029	-0.638	-0.784	-0.737	-0.825	-0.614	-0.950	-0.758
Not selected	NGB00108	-0.990	-0.959	-0.879	-0.946	-0.881	-1.005	-0.943
8	NGB00082	-0.883	-1.093	-1.010	-0.955	-0.972	-0.879	-0.965
9	NGB00059	-0.952	-1.269	-1.518	-0.874	-0.612	-1.251	-1.079
10	NGB00078	-1.617	-1.321	-1.833	-1.317	-1.318	-1.509	-1.486
Mean		0	0	0	0	0	0	
Standard deviation		1	1	1	1	1	1	

YxPHT, YxNOL, YxSTG, YxTBM, YxRWT, YxHI = Yield combination with plant height, number of leaves per plant, stem girth, root weight, and harvest index respectively.

4. Discussion and Conclusions

The significant difference observed among the accessions indicated the existence of variation with respect to the measured traits except for stem girth, and underscored the possibility of selection for improvement. Idehen *et al*. (2018) reported same result for stem girth among 10 accessions of *Amaranthus* spp. On the contrary, Mandal and Dhangrah (2012) studied 17 Amaranthus accessions and observed significant differences among the accessions for all the characters considered including stem girth. Plant height, number of leaves per plant, root biomass and harvest index can be relied upon as important tools in long term selection gain. Gerrano et *al*. (2015) found high phenotypic variability among 32 Amaranthus accessions using plant height, leaf length, leaf width, leaf area, leaf area index, number of leaves, stalk diameter, panicle or inflorescence length, number of primary branches, fresh biomass, dry biomass, harvest index, thousand seed weight and grain yield per plant.

The loadings of plant height, number of leaves per plant, and root weight indicated that these characters chiefly accounted for most of the variation observed among the accessions. This suggests that these traits are crucial in maintaining variability within the breeding population and they should be considered for selection in *Amaranthus* improvement programme. Gerrano *et al*. (2015) reported comparable results on 32 *Amaranthus* species of South African origin.

The positive significant correlation observed between plant height and number of leaves per plant, stem girth, total biomass, root weight and harvest index implied that direct selection for any of the trait could lead to improvement in the other. Gerrano et *al*. (2015) reported that plant height correlated positively with fresh biomass and dry biomass when evaluating the genetic diversity of *Amaranthus* species in South Africa. Thanapornpoonpong *et al*. (2007) also reported a significant and positive relationship between plant height and fresh biomass. Strong positive correlation of stem girth, total and root biomass and stem weight with number of leaves per plant, total biomass and root biomass, stem weight with total biomass, root biomass and harvest index indicated that the use of any of the characters can help to improve selection process in breeding programs. The significant negative correlation of harvest index with plant height and root weight suggested that an attempt to breed for high harvest index will lead to short plant height and less root weight in Amaranthus.

The clustering pattern of accessions into groups showed the phenotypic diversity among the accessions for the different characters studied. Genetic diversity, evidenced by phenotypic variability, is essential in the initiation of a breeding program because when absent, there cannot be meaningful selection and genetic advancement becomes impossible (Govindaraj *et al*., 2014). The accessions within a cluster are closely associated and this suggests that the variability within group could be useful in the selection process for improvement of associated desired traits. Variability between groups could be explored in heterotic breeding where members of a cluster could serve as parents in crosses involving members from distinct clusters. For instance, accessions in Cluster 1 were mostly associated with harvest index, number of leaves per plant, and stem and root weights suggesting the presence of favourable alleles for yield-related traits within the group and a potential to improve these traits via selection. Whereas, individuals in Cluster 2 were generally associated with plant height and stem girth. A cross between members of the two groups could give higher yielding progenies with good standability which would be invaluable to the vegetable industry.

The GYT biplot is useful for multiple-trait-based evaluation of accessions, permitting a graphical ranking of entries based on their levels in combining yield with related traits. Like in the GGE biplot, the GYT polygon is delineated into sectors, with their associated closely-related Y-T combinations, a vertex and other associated accessions (Yan and Frégeau-Reid, 2018). Thus, NGB00019 and NGB00061 were associated with all the Y-T combinations considered in this study which is an indication of superior trait profile. However, NGB00019 was the vertex accession and was thus identified as the best combiner of yield with other traits.

There was sufficient genetic variability among the amaranthus accessions, to permit improvement through selection, with greater chances of success with plant height, number of leaves per plant, and root weight. Harvest index; which is the edible portion of the plant, can be simultaneously improved with plant height, and stem and root weights. Information from principal component analysis, genetic distance estimates, and cluster analysis can

be utilized for parental selection in breeding programmes. Accessions NGB00019, NGB00025, NGB00058, NGB00060, NGB00061, and NGB00112 would be good genetic materials for total biomass, and root and stem weights while NGB00031 and NGB00070 have potentials to improve harvest index, and NGB00005 and the local check would be useful for plant height, number of leaves per plant, and stem girth. NGB00019 followed by NGB00061 had the most diverse trait profile among the evaluated accessions. Further studies involving more traits are required to determine the trait profiles of the remaining accessions used in the study.

Acknowledgements

We are grateful to the National Center for Genetic Resources and Biotechnology, NACGRAB, Ibadan, Nigeria, for providing the seeds used for the study. We thank the staff of the Teaching and Research Farms of the Department of Crop Production and Horticulture, Lagos State Polytechnic, Ikorodu, Nigeria, for assistance during field evaluation.

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Adv. Hort. Sci., 2021 35(3): 285-292 DOI: 10.36253/ahsc-10536

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Investigation of modified WPM medium for the best meristem proliferation of *Corylus avellana* L.

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Key words: Charcoal, Fe-EDDHA, hazelnut, ISSR, micropropagation.

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Citation:
KIVRAK KİRAN S., GALATALİ S., YENİOCAK S., OZKAYA D.E., MERCAN T., GULDAG S., CELİK O., ABDUL GHAFOOR N., KAYA E., 2021 - *Investigation of modified WPM medium for the best meristem proliferation of Corylus avellana L.* - Adv. Hort. Sci., 35(3): 285-292

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 27 February 2021
Accepted for publication 29 June 2021

Abstract: Cultivation of *Corylus avellana L.* in Turkey is performed generally in the northern regions where it is an important source of livelihood for the local farmers. More than 70% of world hazelnut production is supplied by Turkey, but compared with other countries, Turkey's hazelnut production area is quite narrow. In this study was aimed to develop an effective *in vitro* production for seven local cultivars of *C. avellana*. Therefore, WPM medium supplemented with 6-Benzylaminopurine (BAP) was modified by using single or in combination of Fe-EDDHA, $AgNO_3$, H_3BO_3, charcoal and gibberellic acid. In all varieties, the best regeneration rates varying between 68% and 94% were obtained from WPM medium supplemented with 4.4 µM BAP, 27.8 µM Fe-EDDHA and 10g/L Charcoal. Genetic stability of shoots derived from meristem culture using the best medium was analysed using ISSR primers, when the gel images of the PCR products were examined, no polymorphic band was observed in samples collected from seven provinces, and the genetic stability was determined as 100%.

1. Introduction

Corylus avellana L. plants are grown at altitudes in the range of 20-1500 m in the northern parts of the Turkey, they can also be grown in the southern and eastern creeks, and rarely in the western regions (Aydinoglu, 2010). Annual rainfall is balanced according to the seasons and temperatures, above 700 mm rainfall and temperatures ranging from -8°C to -10°C with the highest between 33°C and 36°C are ideal conditions for the production of *C. avellana* L. (Koksal *et al*., 2006). More than 70% of world hazelnut production is produced by Turkey (Zhao *et al*., 2015). However, Turkey's hazelnut production efficiency is very low compared to other countries. The average yield is 179.2 kg/ha in the US, 168.6 kg/ha in Italy whereas in Turkey it is 91.7 kg/ha (Mennan *et al*., 2020; Sen and Kahveci, 2020).

The *in vitro* propagation procedures for clonal multiplication provide the useful alternative tools to traditional propagation of hazelnut cultivars. Development of an efficient procedure for hazelnut micropropagati-

on can also be beneficial for increase of clones, cultivars and varieties resistant to different pathogens such as hazelnut worm (*Curculio nucum*), eastern filbert blight (Anisogramma anomola) and powdery mildew (*Erysiphe corylacearum*) (Bassil *et al*., 1992; Lucas *et al.,* 2018).

The transfer of parts of the plant such as tissue, cell, organ, embryo to *in vitro* environment under aseptic conditions in an artificial nutrient medium for clonal propagation is important to produce a pathogen-free plant with protected germplasms and/or to maintain its secondary metabolites production which otherwise is limited for various reasons is called plant tissue culture (Hayta *et al*., 2017; Espinosa-Leal *et al*., 2018). In addition to the continuous production throughout the year by obtaining thousands of plants with the same form and characteristics as the mother plant in a short time by micropropagation method from plant tissue cultures; superior species resistant to factors such as drought, salinity, acidity, and/or cold can also be produced. Micropropagation involves plant selection, sterilization, *in vitro* transfer, clonal propagation, rooting, conditioning, and transfer to soil. Due to these advantages, micropropagation is one of the most effective methods when compared with other production techniques (Ozudogru *et al*., 2011; Ozudogru and Kaya, 2012).

Different techniques in plant tissue culture can be used such as shoot tip culture by transferring the shoot tips with growth cone including meristematic doom to *in vitro* environment, bud node culture by transferring the axillary or apical buds of the shoots to aseptic conditions, meristem culture by transferring meristem from the meristematic region to the nutrient medium with the help of a stereomicroscope, embryo culture by taking the embryo from the seed template or from the seed and transferring it to the germination medium (Ahmad and Anis, 2007; Usha *et al*., 2007).

Clonal production of plants in a short time is one of the main purposes of plant tissue culture, however, changes may occur in micropropagated plants, all the changes that may occur in the hereditary material are called somaclonal variation, somaclonal variations can be detected by various molecular tests (Samir, 2004; Lattoo *et al*., 2006; Ozudogru *et al*., 2011). Nowadays, genetic differences in the germplasms of many plant species can be detected using molecular markers, non-specific primers are often preferred in determining the differences between germplasms in methods based on the polymerase chain reaction, these primers are particularly advantageous for plant species that have not been previously studied or have a limited number of researches performed on them (Devarumath *et al*., 2002; Carvalho *et al*., 2004; Kaya and Souza, 2017). Although there are a wide variety of molecular marker techniques used nowadays, AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplification of Polymorphic DNA), SSRs (Simple Sequence Repeats), ISSRs (Inter Simple Sequence Repeats) techniques are preferred. The method to be used is selected based on the technical facilities accessible and the purpose of the study (Belaj *et al*., 2003; Kaya, 2015).

The ISSR technique used in many plants is a simple, fast, and low-cost technique, it also stands out for its ability to work with a small amount of template DNA and provides a high level of information. With all these advantages, the ISSR technique is often preferred in determining the difference in germplasm collections (Gilbert *et al*., 1999; Kaya and Souza, 2017).

When there are some limitations (shoot tip necrosis, big contaminations, sensitivity for surface sterilization treatments) causing from plant material such as shoot tips, nodal segments, buds in *in vitro* propagation studies, seeds can be used as starting material and there are many similar studies in the literature such as micropropagation of *Thymus vulgaris* (Ozudogru *et al*., 2011), *Citrus* spp. (Kaya *et al*., 2016), *Musa* spp. (Kaya *et al*., 2020 b). Similarly, embryos isolated from hazelnut seeds were used as starting material in the current study, as there was a major contamination problem. Thus, the optimal growth medium developed for hazelnut these local varieties in the current study will be a model growth medium for future *in vitro* studies such as cryopreservation.

In this study, WPM nutrient medium developed by Lloyd and McCown which commonly used in micropropagation of woody plants in order to develop an effective environment for *in vitro* meristem reproduction of hazelnut plant, which is relatively difficult to micro-propagate due to some limitations was used (Lloyd and McCown, 1980). The main aim of this study was to determine optimal medium combination by using WPM. Seven different cultivars of hazelnuts grown in seven different provinces of Turkey were examined with different combinations of Benzyl Amino Purine and Gibberellic Acid growth regulators, ethylene diamine di-2-hydroxyphenyl acetate ferric

(Fe-EDDHA), Silver nitrate ($AgNO_3$), boric acid (H_3BO_3) and activated charcoal. In addition, the another aim was to confirmation genetic stability of *in vitro* grown plants. For this reason, the genetic stability of the plants growing in *in vitro* conditions that gave the best results in the study was analysed by the ISSR technique.

2. Materials and Methods

In vitro culture initiation and micropropagation

C. avellana L. plant samples (fruits) from Turkey's; Bartin (Guzelcehisar Village, Inkumu location), Düzce (Akcakoca, Yesilkoy Village), Trabzon (Ortahisar, Yanyamac location), Ordu (Ulubey, Inonu location), Samsun (Carsamba, Ordubasi District), Giresun (Eriklimanı Village), and Zonguldak (Eregli, Sarikaya Village) (seven different) provinces were obtained from their respective hazelnut breeders. The fruits were surface sterilized to remove pathogens before transfer to the *in vitro* medium using 70% Ethanol (EtOH) and 30% Commercial Bleach (Domestos, Unilever©). This step was followed by 3-stage internal sterilization (70% EtOH, 10% H_2O_2, and 10% Commercial Bleach) processes applied to the materials separated from their peels with the help of sterile forceps and pliers (Ozudogru *et al*., 2011; Kaya *et al*., 2013, 2016). After the sterilization processes, embryo isolation was performed from *C. avellana* L. samples under a stereomicroscope in a laminar flow cabinet (Fig. 1 a), the isolated embryos were then transferred to WPM semi-solid medium containing 4.44 µM 6-Benzilaminopurin (BA), 30 g/L sucrose and 7 g/L agar for germination (pH 5.8). The germinated embryos were taken into WPM medium supplemented with 4.44 µM BA 30 g L^{-1} sucrose, 7 g L^{-1} agar and 27.8 µM Fe-EDDHA [Ethylenediamine-N, N'-bis (2-hydroxyphenylacetic acid) iron sodium complex] (Fig. 1 b). In order to determine the suitable medium for the hazelnut plant, the meristematic regions of 0.3 - 0.8 mm length of shoots of the seven different hazelnut varieties grown *in vitro* were isolated (Fig. 1c) and transferred to eight different modified WPM mediums (Table 1). The all *in vitro* materials were incubated at standard culture conditions (27±2°C, 16/8 h photoperiod, with cool daylight fluorescent lamps rated at 50 µmol^{-1} m^{-2} s^{-1}).

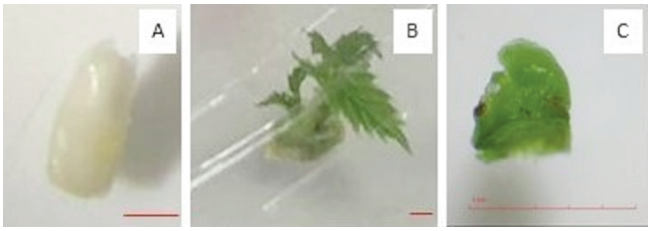

Fig. 1 - The embryo was isolated from fruit of *C. avellana* L. cv. Duzce-Akcakoca (A), the embryo germination on WPM semi-solid medium containing 4.44 µM BAP 30 g L^{-1} sucrose and 7 g L^{-1} agar 27.8 µM Fe-EDDHA (B), the meristem was isolated from *in vitro* grown shoot of *C. avellana* L. cv. Duzce-Akcakoca (C) (Bars 1 mm).

Table 1 - Eight different modified WPM mediums for *in vitro* meristem proliferation of seven *Corylus avellana* L. cultivars

Medium	Plant growth regulator	Chemical
WPM 1	4.44 µM BA	27.8 µM Fe-EDDHA
WPM 2	4.44 µM BA 2.89 µM GA_3	27.8 µM Fe-EDDHA
WPM 3	4.44 µM BA	27.8 µM Fe-EDDHA 5.89 µM $AgNO_3$
WPM 4	4.44 µM BA	27.8 µM Fe-EDDHA 16.17 µM H_3BO_3
WPM 5	4.44 µM BA	27.8 µM Fe-EDDHA 10 g L^{-1} Charcoal
WPM 6	4.44 µM BA 2.89 µM GA_3	27.8 µM Fe-EDDHA 10 g L^{-1} Charcoal
WPM 7	4.44 µM BA	27.8 µM Fe-EDDHA 5.89 µM $AgNO_3$ 10 g L^{-1} Charcoal
WPM 8	4.44 µM BA	27.8 µM Fe-EDDHA 16.17 µM H_3BO_3 10 g L^{-1} Charcoal

Determination of genetic stability

In order to determine the effects of the modified WPM nutrient medium that gives the best regeneration with the meristem culture method on the genetic stability of the plant, DNA isolation was performed manually following the 2× CTAB protocol developed by Ferdous *et al*. (2012). Following the DNA isolation, the protocol developed by Williams *et al*. (1990) was used with the ISSR primers developed by Martins-Lopes *et al*. (2007) [(TC) 8C UBC823; (GA) 8C UBC811; (AG) 8G UBC809; (CA) 8A UBC817; (AG) 8T UBC807; (GA) 8T UBC810] to perform an ISSR PCR reaction. The products obtained from the ISSR PCR were run on an agarose gel electrophoresis containing 1.5% agarose and the bands profiles were visualized under UV-transilluminator. The "Similarity Ratio" formula developed by Sokal and Sneath (1963) was used to

calculate the similarities between the samples in determining the genetic stability or differences as a result of data analysis by scoring the band profiles displayed as a result of ISSR PCR according to the 1/0 (yes/no) principle.

Similarity ratio= Homologous bands/(Homologous bands + Non homologous bands)

The DNA of the samples were obtained from *in vitro* embryo-grown leaves (in WPM medium supplemented with 4.44 µM BA), leaves of shoots that developed after subculture (in WPM medium containing 4.44 µM BA and 27.8 µM Fe-EDDHA), and post-meristem culture grown shoot leaves (in WPM medium containing 4.44 µM BA, 27.8 µM Fe-EDDHA and 10 g L^{-1} charcoal) were used.

Experimental design, data collection, and statistical analysis

For each cultivar, more than 100 embryos of seeds were used for *in vitro* culture establishment. Three Petri dishes (replicates), each containing 10 shoot tips, were used for each *in vitro* proliferation treatment, and each experiment was repeated at least three times. Data of *in vitro* proliferation were recorded 4 weeks after culture initiation and consisted of the percentage of shoot tips that generated at least one elongated shoot. Regeneration percentage was compared by multiple X2 test by the SPSS program (IBM SPSS Statistics 21.0) and statistical analysis was also performed with ANOVA, followed by the LSD test at P≤0.05 (Marascuilo and McSweeney, 1977).

3. Results

After surface sterilization, the embryos germinated in WPM medium containing 4.44 µM BAP were transferred to the regeneration medium, from the samples that were used as starting material, 71% to 85% sterile material was obtained as a result of surface sterilization. The obtained clean materials showed successful regeneration between 81.1% and 100%. Shoot tips grown *in vitro* were subcultured, and reproduced in 30-day periods, and used as the material in meristem culture study. The desired amount of *in vitro* material was obtained from regenerated plants for use in meristem culture applications.

The best regeneration rate from eight different WPM media in different combinations tested for meristem regeneration was obtained from WPM media containing 4.44 µM BAP, 27.8 µM Fe-EDDHA, and 10 gL^{-1} charcoal. The regeneration rate obtained on this medium ranged from 68% to 94% for the seven hazelnut local cultivars tested (Fig. 2).

Polymerase chain reactions were carried out using six different primers to determine the genetic stability of regenerated hazelnut varieties. In order to determine genetic stability, clones of each sample developed from a single embryo were used. PCR products obtained from the PCR using six different ISSR primers were run on an agarose gel and visualized. A total of 324 bands with a length of 120 bp and 2800 bp were scored from the band profiles obtained. In the light of the band profiles obtained, no polymorphic bands were observed in samples collected from the seven provinces, and the genetic stability was determined as 100% (Fig. 3).

4. Discussion and Conclusions

In Turkey, for hazelnut production, the certified seedlings have not still been studied and conventional production does not meet all requirements of Hazelnut plants. Because these plant cultivars can be affected very quickly by climatic conditions and fruit

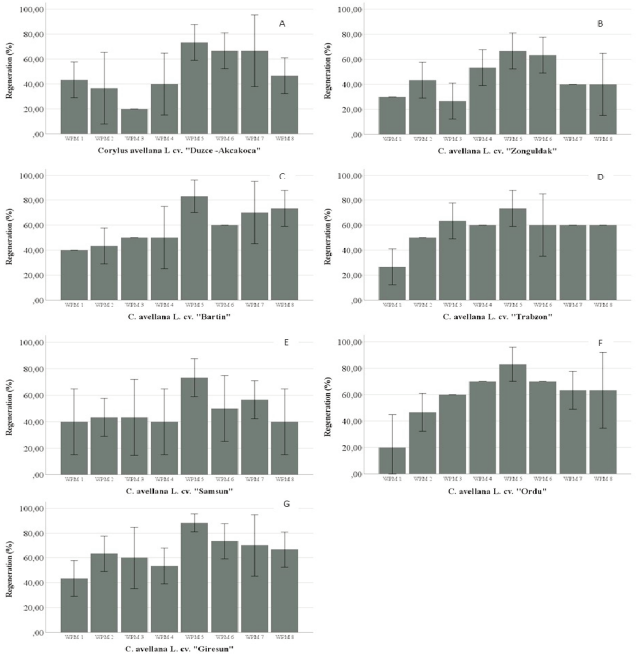

Fig. 2 - The meristem regeneration percentages of seven different cultivars of *C. avellana* L. on eight different modified WPM mediums.

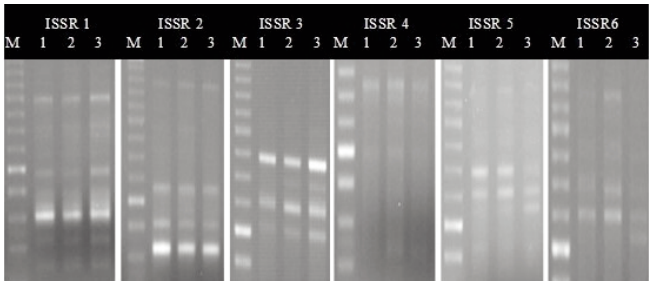

Fig. 3 - The interaction effect of different rootstocks and NO_3:NH_4 ratio on A) number of leaves and B) number of nodes.

yield may decrease accordingly, physical factors such as the reduction in the mineral and organic matter content of the soil, and biological factors such as hazelnut pests and diseases can further decrease its yield. In this case, it is extremely important to examine the factors that cause low productivity and to determine the measures that could eliminate these factors or reduce their effect (Amaral *et al*., 2006; Aydinoglu, 2010; Ciarmiello *et al*., 2014).

Increasing trends in areas such as the development of systems for densely planting tree seedlings, selection of new parents for nutrition programs, development of new cultures in order to better evaluate the existing limited areas, increased the need for modern techniques in fruit tree cultivation. Traditional methods, which were carried out to meet the demand for more high-quality plant material are insufficient for they are both time consuming and insufficient for standard and healthy production, therefore, the application of *in vitro* techniques in fruit growing may be a suitable alternative way to overcome these problems. Developments in plant biotechnology, particularly in the fields of *in vitro* cultures and molecular biology, have been a powerful tool in the control and conservation of plant biodiversity (Withers, 1995). Nowadays, biotechnological methods comprise the most suitable methods for pathogen-free short, medium, and long-term protection of ornamental, medicinal, and aromatic plants as well as woody plant species, which are in danger of extinction and poses rare economical value. *In vitro* conservation methods are especially important for the protection of vegetatively propagated plant species with seeds that are intolerant to desiccation (Engelmann, 2011). In addition to the advantages mentioned above, *in vitro* techniques provide a reliable platform for international plant material exchange, enable large collections using minimal space, provide valuable materials for species recovery, and facilitate molecular research and ecological studies (Tandon *et al*., 2005).

Traditional propagation procedures for hazelnut through shoots is time consuming, limited and inefficient for large scale production. These days, many hazelnut cultivars have been identified for plantation in Turkey and it immediately needs to develop efficient procedures for rapid clonal propagation. The plant tissue culture systems offer an useful tools for large scale production of plantlets (Damiano *et al.*, 2005; Bacchetta *et al*., 2008). The current study aimed to develop and integrated procedure to clonal propagation of Turkish local hazelnut cultivars with optimal combination of growth media supplemented with plant growth regulators/additives in tissue culture techniques.

Meristem culture has been used for the purification of pathogens from plants since the 1930s (Dodds and Roberts, 1986; Nehra and Kartha, 1994). In this technique, the difference in the amount of pathogens in different plant cells is utilized such that the apical and root meristem cells of the plant are very small due to their continuous division characteristics hence the probability of viruses in these cells is very low, therefore, these tissues are highly preferred in the production of pathogen-free and/or disease-free plants (Kaya *et al*., 2020 a). Although it cannot be stated that these parts of the plant are completely free from viruses, the probability can be increased when applying different treatment combinations together (Sherwood, 1993). In this study, meristem culture examinations were carried out using meristematic regions of length between 0.1-0.8 mm extracted from axenic (virus-free) shoots of plants belonging to hazelnut varieties collected from seven different provinces that were grown *in vitro* using clonal propagation. The micro-shoots regenerated *in vitro* were subcultured in 4-6 week periods at standard conditions and the best regeneration rate among the eight different WPM media containing different ingredient combinations tested for meristem regeneration was obtained from WPM medium containing 4.44 µM BAP, 27.8 µM Fe-EDDHA and activated charcoal, and the regeneration rate obtained on this medium was between 68% and 94% for seven different hazelnut local cultivars tested.

The iron component of medium is an essential micronutrient for Hazelnut *in vitro* cultures and these cultures can also be very sensitive to the iron source and concentration. In *in vitro* cultures of hazelnut, beneficial effect of Fe-EDDHA as source of iron, have

been reported in previous studies (Nas and Read, 2001; Garrison *et al*., 2013). In the current study, the significant multiplication rates in hazelnut local cultivars were achieved by using medium supplemented with Fe-EDDHA as iron source.

In the current study, the media supplemented with activated charcoal showed the beneficial effect on in vitro cultures of all hazelnut local cultivars. In *in vitro* cultures, because of their absorbtion ability (growth regulators, inhibitors, other compounds), the activated charcoal can promote growth (Pan and Staden, 1998).

Morphological differences that make up biological and pharmaceutical characters are under the influence of environmental and/or genetic factors (Hay and Waterman, 1995). In recent years, DNA-based molecular markers have been used to determine genetic differences in the germplasms of many plant species, the use of various DNA markers in plant research has become increasingly common as they provide a larger number of markers than allozymes. In various DNA-based PCR methods, mostly non-specific primers are used to determine the differences between (and within) species. These methods, which are preferred because they do not require sequence knowledge in target species, are particularly suitable for plant varieties on which none or very little research has been done before (Walton, 1993).

In this study, the genetic stability of hazelnut samples collected from seven different provinces after meristem culture was evaluated by PCR using six different ISSR primers. Polymorphism was not detected in the cultivars as a result of visualizing the PCR products in agarose gel. In this context, we can say that the nutrient medium optimized for meristem culture does not have a negative effect on the genetic stability of the hazelnut varieties tested.

It is known that plants propagated *in vitro* by shoot tip, axillary bud, and meristems, maintain their genetic stability during cell division and differentiation under *in vitro* conditions (Shenoy and Vasil, 1992; Ostry *et al*., 1994). However, it should not be overlooked as there is a risk of alteration of genetic stability induced during the *in vitro* applications (types and concentrations of growth regulators, nutrient conditions). There are many studies in the literature regarding this (Rani *et al*., 1995; Giménez *et al*., 2001), these changes in genetic stability are often inherited and can be passed down from generation to generation during micropropagation (Breiman *et al*., 1987).

Acknowledgements

This study was supported by Mugla Sitki Kocman University, Scientific Research Projects Coordination Unit (Mugla, Turkey, MSKU-BAP, Project Number: 17-057).

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Adv. Hort. Sci., 2021 35(3): 293-303 DOI: 10.36253/ahsc-10740

AHS
Advances in Horticultural Science

Biochemical changes in pear fruits during storage at ambient conditions

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Key words: Cellulase, minerals, 'Patharnakh', pectinmethylestrase, polygalacturonase, 'Punjab Beauty', quality attributes, ripening physiology, sugars.

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Citation:
KAUR A., SHARMA S., SINGH N.P., 2021 - *Biochemical changes in pear fruits during storage at ambient conditions.* - Adv. Hort. Sci., 35(3): 293-303

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 31 March 2021
Accepted for publication 3 August 2021

Abstract: 'Patharnakh' (PN) (*Pyrus pyrifolia* Burm. Nakai) and 'Punjab Beauty' (PB) [*Pyrus communis* L. × *Pyrus pyrifolia* Burm. (Nakai)] are leading low-chill pear cultivars of subtropics of India. Diurnal temperature and relative humidity during fruit harvest period is high which considerably affect the shelf life of fruits. Fruits of 'PN' and 'PB' pear harvested at physiological maturity were stored for 12 days at ambient temperature and effects of storage temperature on physical and qualitative parameters were studied. Both cultivars showed reduction in fruit weight and firmness, reducing sugars, sucrose, starch and pectin content. However, total soluble solids and juice acid content increased during storage. Sucrose synthase activity and sucrose content showed significant positive correlation in 'PN' cultivar. Activities of fruit softening enzymes such as polygalacturonase (PG) and cellulase was enhanced; whereas, pectinmethylesterase (PME) was reduced during storage. Fruit firmness was negatively correlated with PG in both the cultivars. In 'PN' cultivar, fruit firmness was positively correlated with cellulase and negatively with PME enzyme but reverse trend was observed in 'PB' cultivar. Fruit minerals content didn't show any substantial disparities in both the cultivars during storage. 'Patharnakh' and 'Punjab Beauty' fruits maintain desirable quality parameters up to 6-9 days and 3-6 days, respectively, during storage at ambient conditions.

1. Introduction

Pear (*P*yrus spp.) ranks second next to apple fruit crop in the world in terms of area, production and varietal wealth among temperate fruits. It belongs to the family *Rosaceae* and sub family *Pomoideae*. In India, it is cultivated in Himachal Pradesh, Uttarakhand, Punjab, Jammu & Kashmir and some parts of Assam and Nilgiris hills. In Punjab province of North-West India, the area under pear cultivation is dominated by low chill cultivar 'Patharnakh' that belongs to Oriental or Sand pear group (*Pyrus pyrifolia* Burm. Nakai) and semi soft pear cultivar 'Punjab Beauty, a hybrid between *Pyrus communis* L. × *Pyrus pyrifolia* Burm. (Nakai) (Sharma and

Singh, 2011) and fruits are harvested at physiological maturity during IInd fortnight of July. Diurnal temperature and relative humidity during fruit harvest period is high which considerably affect the shelf life of fruits. It is documented that pear fruits have post-harvest shelf-life of about 10 days at ambient conditions (25-30°C) and quality related parameters are reduced rapidly during storage (Nath *et al*., 2011). After harvest, consumers' preference and market price depends on fruit's attractive colour, flavor, taste, aroma and firmness. The variability observed in volatile organic compounds, physico-chemical and sensory parameters can be used to understand the ripening behavior of pear cultivars (Taiti *et al*., 2017). It is suggested that fruits should be harvested at optimal physiological maturity and kept under optimal storage conditions to enhance the shelf life of fruits (Hafez *et al.,* 2019). Fruit quality deteriorates after harvest due to rapid change in respiration, activity of cell wall degradation enzymes and infestation of pathogens during transportation and storage (Ge *et al*., 2017).

The quality related attributes constantly depend on the storage temperature which primarily affect fruit freshness and shelf life. Quantification of organic acids and soluble sugars (sucrose, glucose and fructose) are correlated to the production of quality fruits (Itai and Tanahashi, 2008). Sugars content in pear fruit improve during early storage period and further decline with the advancement of storage period at ambient conditions is due to fermentation into alcoholic content (Kaur and Dhillon, 2015). Softening is associated with the degradation of cell wall polysaccharides and biosynthesis of cellulase, polygalacturonase and pectin methyl esterase enzymes (Zhou *et al*., 2011). Fruit minerals content can also modify the quality attributes and storability (Saquet *et al*., 2019). It is well recognized fact that pome fruits are harvested at proper maturity stage and must be stored under explicit low temperature to extend the shelf life without exhibiting any deterioration in fruit quality attributes (Itai *et al*., 2015; Yu *et al*., 2016). However, less information is available on the ripening behavior of pear fruits harvested at physiological maturity (135 DAFS) and kept in ambient conditions and subsequently, its effect on the biochemical composition during storage. Therefore, the study was performed to record the changes in physical characteristics, sugars composition, activities of hydrolytic fruit softening enzymes and minerals profile during storage of pear fruits at ambient conditions.

2. Materials and Methods

Experimental procedure

Fruits of 'Patharnakh' 'PN' and 'Punjab Beauty' 'PB' cultivars (Fig. 1) grafted on Kainth rootstock (*Pyrus pashia*) were harvested during IInd fortnight of July (135-145 days after fruit set; DAFS) from the orchard situated at Research Farm, Department of Fruit Science, Punjab Agricultural University, Ludhiana (India) (30.90° N, 75.86° E). Fifteen fruits/replication free from any type of visual injury and bruises of each cultivar were washed with sodium hypochlorite 4% (2.5 ml L^{-1}) solution for 5 minutes (PAU, 2020). Fruits were dried in shade and packed in three ply corrugated fiberboard with 5% perforation and stored at ambient temperature (28±2°C). Physico-chemical parameters, physiological changes and enzymatic activities were estimated after the intervals of 0, 3, 6, 9 and 12 days of storage.

Physiological loss in weight (PLW)

Fruits stored at ambient temperature were weighed before storage and at a subsequent storage interval. The values were expressed as PLW (%) (Singh *et al*., 2021).

Fruit firmness

Fruit firmness was measured at every storage

Fig. 1 - Fruits of 'PatharNakh' (top) and 'Punjab Beauty' (bottom) cultivars at physiological maturity.

interval with penetrometer (Model No. FT-327, QA Supplies LLC, USA) and values were expressed lbs (Mahajan *et al*., 2010).

Total soluble solids, Titratable acidity and fruit color coordinates

Titratable acidity (TA) was determined with titration method described by Ranganna (2007) and expressed as percent of maleic acid. Fruit color coordinates (L*, a*, b*, C* and h*) were randomly measured on two opposite sites at fruit equator using Color Flex Spectrophotometer (Hunter Lab Color Flex, Hunter Associates Inc., Reston, VA, USA). These coordinates were expressed in CIE units (Hunter, 1975).

Sugars

Fruit pulp was homogenized with 80% ethanol and refluxed twice for 20 min. The supernatants were pooled to evaporate ethanol and volume was made 10 ml with distilled water. This extract was used for the estimation of reducing sugars, fructose and sucrose by the methods already described by Kaur *et al*. (2018). For the estimation of fructose, 0.1% resorcinol reagent and 30% HCl were added to sugar extract and color intensity was recorded at 540 nm. Estimation of sucrose was done using the same procedure except that free fructose was destroyed by treating the sample with 6% KOH and the absorbance was measured at 490 nm. The residue left after sugar extraction was dried and treated with perchloric acid to hydrolyze starch into simpler sugars and were estimated using the method of Dubois *et al*. (1956).

Sucrose metabolizing enzymes

Enzymes viz. sucrose synthase (SS), sucrose phosphate synthase (SPS) and invertases (acid and neutral) were extracted from fruit pulp using HEPES-NaOH buffer (pH 7.5) and assayed by the methods described by Asthir and Singh (1995) and Singh *et al*. (1978). For SS assay, 0.1 ml fructose (150 mM), 0.1 ml UDPG solution (20 mM) and 0.2 ml enzyme extract were incubated for 30 min at 37°C, followed by addition of 0.1 ml of 30% KOH and contents were boiled. Added 1 ml resorcinol reagent and 3 ml of HCl and tubes were kept for 10 min at 80°C. After cooling the tubes, the absorbance was noted at 490 nm. For SPS assay, fructose-6-phosphate (150 mM) was used as substrate and enzyme activity was expressed as mg sucrose formed g^{-1} min^{-1} fresh weight (fw). For acid invertase, 0.6 ml sodium acetate buffer (0.2 M, pH 4.8), 0.2 ml sucrose (50 mM) and 0.2 ml of enzyme extract were incubated for 1 h at 37 °C followed by addition of 1 ml Nelson reagent C. Contents were boiled for 20 min and then 1 ml Nelson reagent D and 7 ml of distilled water was added and mixed well. Absorbance was read at 510 nm. Sodium phosphate buffer (0.2 M, pH 7.5) was used for neutral invertase assay in place of acetate buffer and rest of the procedure was same as described for acid invertase. Invertase activity was expressed as mg glucose formed min^{-1} g^{-1} fw.

Pectin content and cell wall degrading enzymes

For pectin content, 50 g fruit pulp and 50 ml of 0.01 N HCl were boiled for 30 min and supernatant was collected. The process was repeated twice using 0.05 N and 0.3 N HCl and volume of filtrate was made to 100 ml. Two ml of diluted extract was neutralized using 1 N NaOH. To this, calcium chloride was added next day for precipitation. Precipitates were collected, weighed and % calcium pectate content was calculated (Okimasu, 1956). Fruit pulp was crushed with 0.1 M sodium acetate buffer (pH 5.2) and supernatant was used for the assay of cellulase and polygalacturonase enzymes. For cellulase, 1 ml of 0.1 M sodium acetate buffer (pH 5.2), 1 ml of 0.5% carboxymethyl cellulose (prepared in buffer) and 1 ml of enzyme extract were incubated for 1 h at 55 °C, one ml of dintrosalicyclic acid was added to terminate the reaction. The contents were boiled for 10 min and absorbance recorded at 560 nm. Enzyme activity of cellulase was expressed as mg glucose released min^{-1} g^{-1} fw. Pectic acid (0.5%) was used as substrate for PG assay and enzyme activity was expressed as mg galacturonic acid released min^{-1} g^{-1} fw (Malik and Singh, 1980). Fruit tissue was crushed with 0.1 M citrate phosphate buffer (pH 5.0) and supernatant obtained was used for PME enzyme assay. For reaction, 2 ml of 1% pectin, 2 ml of 0.1 M citrate phosphate buffer (pH 5.0) and 1 ml of enzyme extract were incubated at 35 °C. From this reaction mixture, 1 ml was pipetted out at 0 and 1 h of the incubation and titrated against 0.005 N NaOH. The PME activity was expressed as milliequivalents of methoxyl groups released min^{-1} by 1 ml of enzyme (Balaban *et al.*, 1991).

Mineral's analysis

For nitrogen (N) estimation, dried powder of fruits was digested with H_2SO_4 and content were determined using Kjeldahl method (Gehrke *et al*., 1972). Phosphorus (P) and potassium (K) in fruit samples were digested with a mixture of nitric acid and per-

chloric acid. P estimation was done by the method described by Jackson (1973) and K by flame photometric method (AOAC, 1990). Nutrients like Ca, Mg, Cu, Zn, Fe and Mn were determined using atomic absorption spectrophotometer (Perkin Elmer Analyst 200). The instrument optimization, calibration and elemental analysis were carried out using WinLab32 software as described by Bradfield and Spencer (1965).

Statistical analysis

The experiment was conducted during the year 2020 in a complete randomized design with four replications. Two hundred and forty fruits of each cultivar for different storage intervals were stored at ambient temperature. A lot of 60 fruits for each storage interval with 15 fruits/replication were stored in cardboard boxes. The data was analyzed by one-way analysis of variance. The differences were considered statistically significant at the level P value of < 0.05 using software CPCS1 developed by PAU, Ludhiana and WASP 2.0. Experimental data was represented as mean ± standard error. The data were subjected to Pearson's correlation analysis to assess the relationship between attributes. Principal component analysis (PCA) was used to examine the interrelations between different quality parameters.

3. Results

Physical characteristics

Physiological loss in Weight (PLW) of 'Patharnakh' (PN) and 'Punjab Beauty' (PB) pear cultivars increased during different storage intervals and the higher rate up to 4.75 to 8.18 % was noted in 'PB' between 6 to 9 days compared to 3.21 to 4.16 % in 'PN' at ambient storage conditions (Fig. 2A). The values of reduction in fruit firmness were increased with advancement of the storage period in both the cultivars. The rate of softening of 'PN' fruits was lower than that of 'PB' fruits and values were higher between 6 to 9 days in 'PN' and 3-6 days in 'PB' cultivar (Fig. 2B). During storage, values ranged from 11.6 lbs at 0 day to 9.35 lbs at 12 days in 'PN' and 10.75 lbs at 0 day to 8.38 lbs at 12 days in 'PB'.

Total soluble solids and Titratable acidity

TSS content increased in 'PB' fruits during storage with the mean value of 14.97° Brix and a significant rise in values was recorded from 13.39° Brix at 3 DAS to 16.98° Brix at 9 DAS (Fig. 2C). 'PN' cultivar showed significant variations in TSS content up to 6 days after storage and values varied from 11.05° Brix to 11.63° Brix. There was a significant increase in juice acid content from 3 DAS to 9 DAS and then values remained almost comparable until 12 days of storage in both the cultivars (Fig. 2D). 'PB' showed higher acidity values at all the storage intervals as compared to 'PN' cultivar.

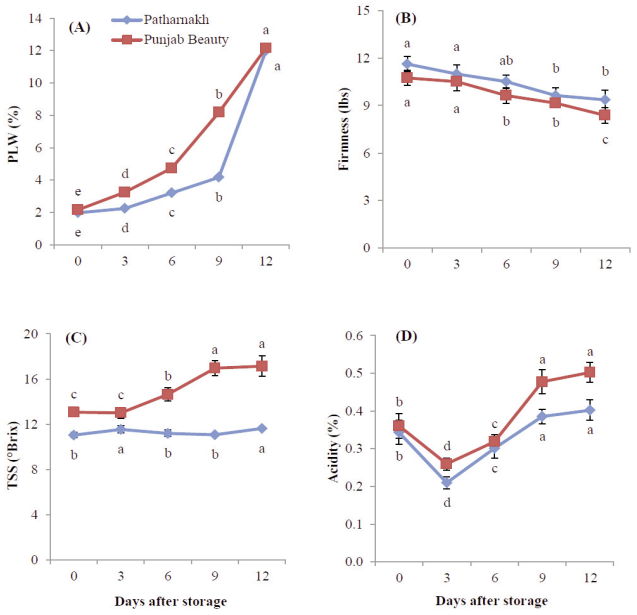

Fig. 2 - Changes in physiological loss in weight (A), fruit firmness (B), TSS (C) and titratable acidity (D) of pear fruits during storage at ambient conditions. Vertical bars represent ± SE of means for 4 replicates. Different letters indicate the significant differences among storage periods according to WASP 2.0 (P≤0.05).

Fruit color

Color coordinates depicting peel color where L* expresses as lightness, a* positive value measures the red intensity and negative value as green color; b* positive value measures yellow color intensity. The value of b* coordinate was improved in both the pear cultivars during storage being highest in 'PB' and lowest in 'PN' cultivar. However, hue angle (h*) showed the reverse trend (Table 1). Significant improvement in a* values from 9 to 12 DAS in 'PN' cultivar was observed; however, other color coordinates showed non-significant variations when the storage period was increased from 3 to 12 days. Initial negative a*values indicated greener colour at zero day as compared to 12 days of storage in 'PN' cultivar.

Table 1 - Changes in fruit color coordinates of pear fruits during storage at ambient conditions

Fruit color	Days after storage					CD (P<0.05)
	0	3	6	9	12	
'Patharnakh' (PN)						
L*	59.00 ± 4.24	61.12 ± 1.91	64.61 ± 2.10	66.07 ± 2.54	67.20 ± 2.19	NS
a*	-1.50 ± 1.22	-2.26 ± 1.05	-0.83 ± 1.62	0.17 ± 0.58	3.24 ± 1.40	1.86
b*	41.54 ± 2.65	40.90 ± 2.12	43.90 ± 1.36	46.09 ± 1.03	50.37 ± 1.74	NS
C*	41.58 ± 2.68	40.97 ± 2.16	43.92 ± 1.38	46.09 ± 1.03	50.49 ± 1.69	NS
h*	92.00 ± 1.59	93.13 ± 1.37	91.03 ± 2.09	89.78 ± 0.72	86.30 ± 1.66	NS
'Punjab Beauty' (PB)						
L*	65.34 ± 1.52	63.49 ± 2.42	63.44 ± 1.72	68.45 ± 0.82	66.67 ± 1.17	NS
a*	3.59 ± 2.35	4.49 ± 1.16	4.14 ± 2.74	1.88 ± 1.19	-0.89 ± 0.54	NS
b*	41.38 ± 1.58	43.25 ± 2.79	43.09 ± 1.07	47.84 ± 1.39	52.16 ± 2.00	NS
C*	41.57 ± 1.66	43.49 ± 2.85	43.35 ± 1.20	47.88 ± 1.39	52.16 ± 2.00	NS
h*	94.93 ± 3.14	95.89 ± 1.32	95.42 ± 3.59	92.25 ± 1.44	90.96 ± 0.55	NS

Carbohydrate composition and Sucrose metabolizing enzymes

Reducing sugars increased up to 3 DAS in 'PN' and 6 DAS in 'PB' fruits and then declined during advanced storage period (Fig. 3A). Fructose content increased in pear fruits from harvest to 6 DAS and later showed a declining trend up to 12 DAS in both the cultivars (Fig. 3B). In 'PB' fruits, sucrose content did not show any differences until 9 days of storage and values were declined at 12 DAS (Fig. 3C). Starch content increased initially until 3 days and then showed declined trend up to final storage interval (Fig. 3D). Both starch and sucrose content improved up to 6 DAS and a decrease in its content was observed from 6 to 12 DAS in 'PN' fruits.

Sucrose synthase (SS) enzyme showed fluctuation in values in both the cultivars with the advancement of storage period (Fig. 4A). After 12 days of storage; SS activity was about 2-fold higher in 'PB' than 'PN' fruits. In 'PN' cultivar, sucrose phosphate synthase

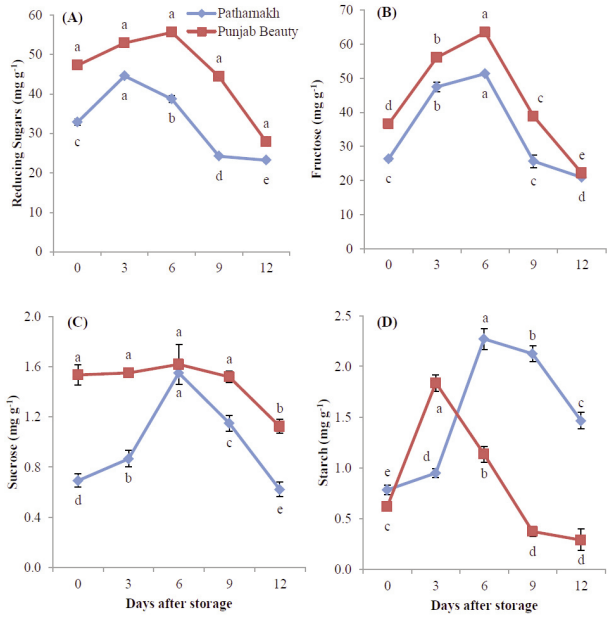

Fig. 3 - Changes in reducing sugars (A), fructose (B), sucrose (C) and starch (D) content of pear fruits during storage at ambient conditions. Vertical bars represent ± SE of means for 4 replicates. Different letters indicate the significant differences among storage periods according to WASP 2.0 (P≤0.05).

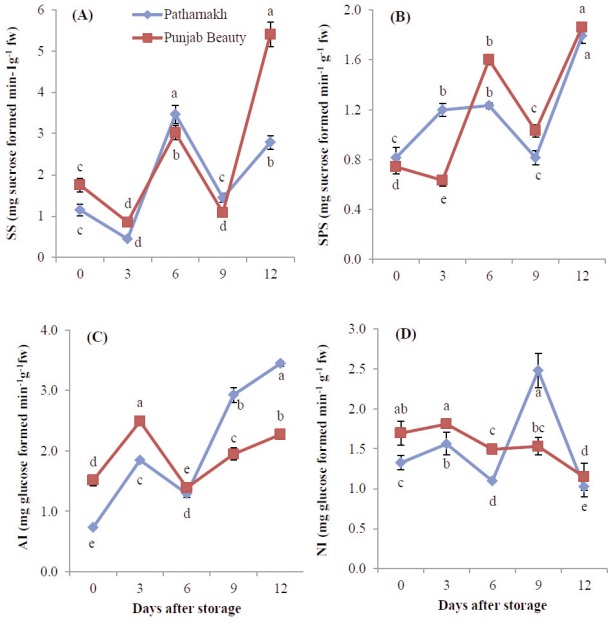

Fig. 4 - Variations in activities of sucrose metabolizing enzymes: SS (A), SPS (B), AI (C) and NI (D) of pear fruits during storage at ambient conditions. Vertical bars represent ± SE of means for 4 replicates. Different letters indicate the significant differences among storage periods according to WASP 2.0 (P≤0.05).

(SPS) activity increased at 3 DAS and exhibited a steady variation with less effectiveness until 6 DAS. In 'PB' cultivar, SPS activity during initial storage period decreased significantly and later showed an upsurge to 6th DAS by 1.5-fold from the initial values and comparably had higher values than 'PN' cultivar. At 6 DAS, both cultivars showed a decline in SPS enzyme activity up to 9 days of storage followed by an upsurge up to 12 DAS (Fig. 4B). Acid invertase (AI) activity increased from 0 to 3 DAS in both the cultivars and subsequently declined at 6 DAS followed by a significant enhancement with progression in storage at ambient temperature (Fig. 4C). Neutral Invertase (NI) activity increased up to 3 days of storage followed by a declining trend after 6 days of storage in both the cultivars. In 'PN' cultivar, NI activity increased until 9 DAS but decreased progressively afterwards. In 'PB' cultivar, NI activity decreased from 3 to 12 DAS (Fig. 4D). In both the cultivars, reducing sugars, fructose and sucrose attributes were correlated positively (data not shown). These sugars presented non-significant negative relationships with PLW except in reducing sugars with PLW (r= -0.483; p ≤ 0.05). In 'PN' cultivar, substantially positive correlation between sucrose accumulation and SS activity (r= 0.46; p≤0.05) and non-significant correlation with SPS (r= 0.09) was observed (Table 2). In 'PB' cultivar, sucrose exhibited negative correlation with SS (r= -0.73, p≤0.01) and SPS (r= -0.54, p≤0.05). AI activity was non-significantly and negatively correlated with sucrose accumulation in 'PN' (r= -0.38) and 'PB' (r= -0.43) cultivar. NI activity and sucrose content were negatively correlated in both pear cultivars.

Pectin and cell wall degrading enzymes

Total pectin content was decreased significantly in both the cultivars during storage (Fig. 5A). PG activity increased significantly in both the cultivars during progression of storage period and values were 1.54 and 2.12-fold higher during last storage period compared to harvest stage in 'PN' and 'PB', cultivars, respectively (Fig. 5B). Cellulase activity also enhanced in pear fruits during storage but showed a significant declining trend from 6 to 12 days of storage in both the cultivars under ambient conditions (Fig. 5C). PME activity was 1.18 ('PN') and 1.24-fold ('PB') lower until 6 days of storage period (Fig. 5D). Thereafter, an increment in PME activity up to 12 DAS was noticed in both the cultivars.

In 'PB', fruit firmness was negatively correlated to cellulase (r= -0.632) and PG (r= -0.857) activities and values were significant at 1% level of significance. PME activity was positively correlated to firmness in these fruits (r= 0.450) at 5% level of significance dur-

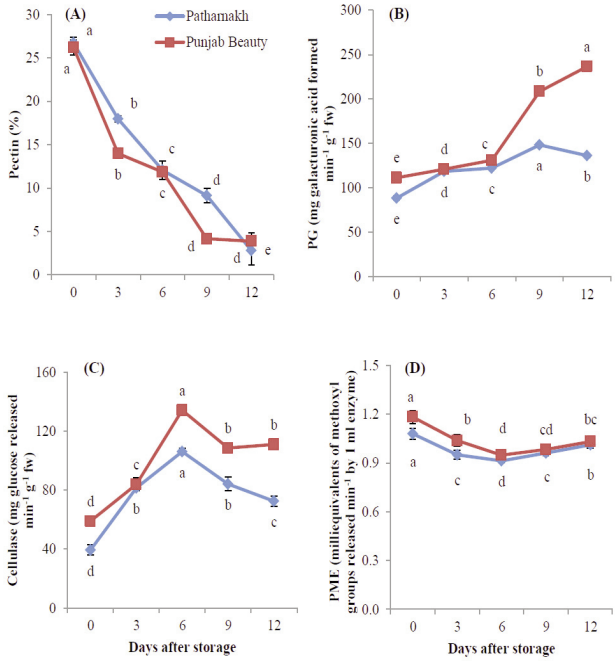

Fig. 5 - Changes in activities of cell wall degrading enzymes as Pectin (A), PG (B), cellulase (C) and PME (D) in pear fruits during storage at ambient conditions. Vertical bars represent ± SE of means for 4 replicates. Different letters indicate the significant differences among storage periods according to WASP 2.0 (P≤0.05).

Table 2 - Correlation between sucrose metabolizing enzymes and sucrose accumulation in pear cultivars during storage

Traits	'Patharnakh'				'Punjab Beauty'			
	Sucrose	SS	SPS	AI	Sucrose	SS	SPS	AI
Sucrose synthase	0.460*				-0.730*			
Sucrose phospate synthase	0.085	0.766**			-0.543*	0.632*		
Acid invertase	-0.376	0.405	0.529*		-0.427	0.486*	-0.109	
Neutral invertase	-0.372	-0.053*	-0.617**	0.267	-0.723**	-0.597**	-0.835**	-0.024

* Correlation is significant at the p≤0.05.
** Correlation is significant at p≤0.01.

ing storage at ambient conditions. In 'PN' fruits, PG activity showed a significant negative correlation with fruit firmness (r= -0.738, P≤0.01) and PME (r= -0.523, P≤0.05) and a positive correlation with cellulase enzyme (r= 0.624, P≤0.01).

Minerals

Nitrogen content decreased significantly at 3 DAS by 1.6-fold in both the cultivars and then increased from 6 to 12 DAS (Table 3). Phosphorus content in both the pear cultivars varied non-significantly during storage. Potassium content in 'PN' significantly increased between 3 to 6 days of storage period and then values remained higher until 12 DAS in both the cultivars. Magnesium content in both the cultivars showed almost similar pattern during storage intervals and significantly lower values at 12 DAS in 'PN' and at 9 DAS in 'PB' fruits were observed. Calcium content in fruits of both the cultivars enhanced significantly until 3 DAS. Iron and zinc content decreased significantly in both the cultivars from harvest to 12 days of storage periods under ambient conditions (Table 4); whereas, manganese content displayed a reverse trend in 'PB' fruits. Copper content was substantially lower during different storage intervals in comparison to harvest stage in both the cultivars.

Principal component analysis (PCA)

Biplot for PC1 and PC2 in pear fruits are given in figure 6. The results showed that first two components explained 62.8% and 71.4% of the total variability in 'PN' and 'PB' cultivars, respectively. In 'PN' cultivar, PC1 includes sucrose, cellulase, starch, PG, AI and acidity attributes which explained 35.9% of total variability. PC2 comprises reducing sugars, fructose, firmness, pectin, and PME parameters and showed total variability of about 26.9% (Fig. 6A). In 'PB' cultivar, PC1 includes cellulase, SPS, TSS, PG and acidity characteristics that described 51.8% of total variability. PC2 comprises reducing sugars, fructose, sucrose, starch, NI, firmness, pectin and PME that described 19.6% of the total variability in physicochemical parameters in 'PB' cultivar during storage at ambient conditions (Fig. 6B).

4. Discussion and Conclusions

Physiological loss in weight (PLW) consists of metabolic activities, respiration and transpiration,

Table 3 - Macrominerals (%) content in pear fruits during storage at ambient conditions

Days after storage	N		P		K		Mg		Ca	
	'Patharnakh'	'Punjab Beauty'	'Patharnakh'	'Punjab Beauty'	'Patharnakh'	'Punjab Beauty'	'Patharnakh'	'Punjab Beauty'	'Patharnakh'	'Punjab Beauty'
0	0.11 ± 0.01	0.13 ± 0.01	2.95 ± 0.09	2.68 ± 0.24	0.93 ± 0.06	1.07 ± 0.06	1.48 ± 0.02	1.45 ± 0.01	0.57 ± 0.02	0.47 ± 0.03
3	0.07 ± 0.00	0.08 ± 0.00	2.91 ± 0.14	2.48 ± 0.20	0.83 ± 0.06	1.07 ± 0.06	1.49 ± 0.02	1.42 ± 0.04	0.68 ± 0.02	0.62 ± 0.03
6	0.12 ± 0.01	0.09 ± 0.01	2.88 ± 0.14	2.65 ± 0.05	1.20 ± 0.00	1.03 ± 0.06	1.48 ± 0.02	1.44 ± 0.03	0.39 ± 0.02	0.33 ± 0.03
9	0.12 ± 0.00	0.12 ± 0.01	2.74 ± 0.11	2.58 ± 0.11	1.17 ± 0.06	1.20 ± 0.10	1.43 ± 0.04	1.19 ± 0.05	0.47 ± 0.02	0.32 ± 0.01
12	0.13 ± 0.00	0.13 ± 0.00	2.72 ± 0.12	2.42 ± 0.09	1.40 ± 0.10	1.17 ± 0.06	1.30 ± 0.02	1.36 ± 0.07	0.28 ± 0.00	0.25 ± 0.01
Mean	0.11 ± 0.00	0.11 ± 0.01	2.84 ± 0.12	2.56 ± 0.14	1.11 ± 0.05	1.11 ± 0.07	1.43 ± 0.02	1.37 ± 0.04	0.48 ± 0.02	0.40 ± 0.02
CD(P≤0.05)	0.01	0.02	NS	NS	0.16	NS	0.05	0.08	0.04	0.05

Table 4 - Microminerals (mg kg^{-1} dw) content in pear fruits during storage at ambient conditions

Days after storage	Fe		Zn		Mn		Cu	
	'Patharnakh'	'Punjab Beauty'	'Patharnakh'	'Punjab Beauty'	'Patharnakh'	'Punjab Beauty'	'Patharnakh'	'Punjab Beauty'
0	83.73 ± 2.87	73.80 ± 2.51	109.13 ± 5.14	188.1 ± 7.69	27.33 ± 0.31	43.40 ± 0.40	94.00 ± 1.40	201.3 ± 6.07
3	30.47 ± 2.21	37.47 ± 3.95	38.13 ± 1.62	51.27 ± 4.57	28.27 ± 1.62	44.20 ± 0.92	27.73 ± 1.42	52.27 ± 0.50
6	26.67 ± 0.95	26.20 ± 0.69	52.60 ± 2.27	35.53 ± 1.81	30.07 ± 1.72	58.13 ± 3.51	43.07 ± 1.94	31.00 ± 2.31
9	21.53 ± 2.12	1.27 ± 0.12	38.40 ± 3.94	45.73 ± 1.89	37.13 ± 1.33	83.80 ± 1.44	27.47 ± 1.55	44.87 ± 2.96
12	4.47 ± 0.42	0.40 ± 0.20	54.47 ± 5.62	48.93 ± 4.10	31.93 ± 3.19	87.20 ± 4.61	58.80 ± 1.77	50.87 ± 5.03
Mean	33.37 ± 1.71	27.83 ± 1.49	58.55 ± 3.72	73.92 ± 4.01	30.95 ± 1.63	63.35 ± 2.18	50.21 ± 1.62	76.05 ± 3.37
$CD_{0.05}$	3.52	3.85	7.34	8.30	3.41	4.92	2.97	7.11

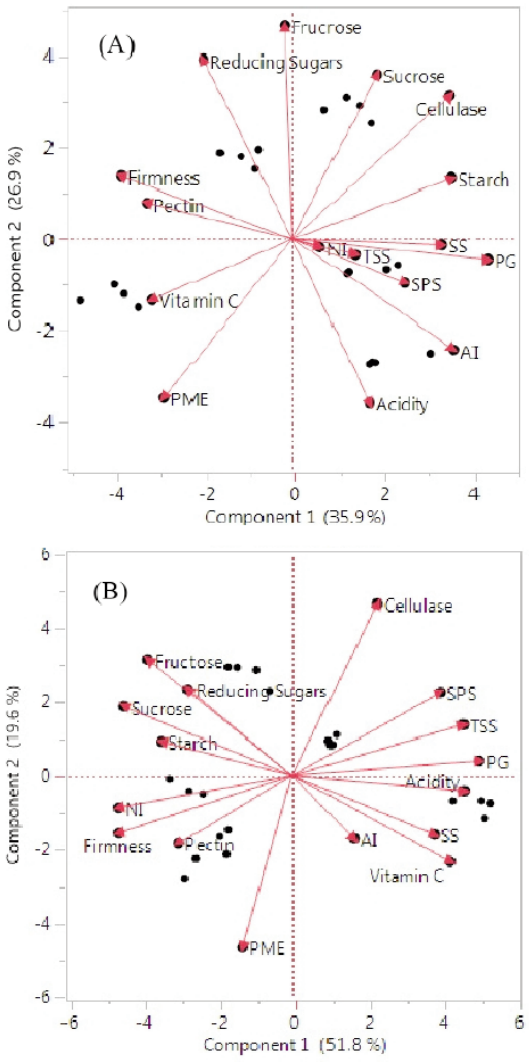

Fig. 6 - Biplot for quality parameters in pear fruits of 'Patharnakh' (A) and 'Punjab Beauty' (B) cultivars during storage at ambient conditions.

water pressure gradient between fruit tissues, environment, stage of ripening as well as storage temperature (Ma *et al*., 2014; Hafez *et al*., 2019). It acts as a detrimental factor to aggravate the fruit freshness, which might be associated with loss of moisture from the tissue (Barman *et al.,* 2014). A nonsignificant relationship between SSC and weight loss in PatharNakh pear during storage was reported by Kaur *et al.* (2019). Fruit firmness is considered as an important index of texture and storage life of pears. Bhat *et al.* (2012) reported a significant reduction in pear fruit firmness with the lowest value after 15 days of storage. Softening of pear fruit during storage could be partly attributed to an increase in depolymerization and degradation of the cell-wall polysaccharides containing pectin, hemicellulose, and cellulose; and loss of moisture (Nath *et al*., 2011). Charoenchongsuk *et al*. (2015) observed a slight variation in Hunter values and hue angle of 'La France' pears during storage. Although L*, a*, b* and C* values showed an increasing trend with storage but these values are not considered for maturity indices of pear fruits. Increment in TSS content may be due to breakdown of organic polymers into simple sugars as reported by Mahajan and Singh (2014) or dehydration of fruits and transformation of pectic substances (Dave *et al*., 2017). Titratable acid content of fruit helps in keeping the fruit taste and flavor (Sajid *et al*., 2019). The increase in TA during storage may be due to conversion of sugars to organic acids and their utilization as a source of energy. Similar findings have been reported by Piga *et al*. (2003) in Cactus pear and 'Bartlett' pear (Bhat *et al*., 2012) during storage.

The reduction in sugars is characterized by higher respiration during storage; whereas, sugars and acids are readily used as substrates for metabolic processes (Ackermann *et al*., 1992) or fermentation of overripe fruits which converts sugars into alcohol (Kaur and Dhillon, 2015). The decrease in fructose content with advanced storage has also been reported by Chen *et al*. (2006) and Dave *et al*. (2017). SS, SPS and invertases enzymes substantially regulate sucrose synthesis in plants. These findings are corroborated with the observations reported by Chen *et al*. (2019) and they explained that activities of SS and SPS increased during initial storage period. Duan *et al*. (2019) reported that activities of SS cleavage and synthesis of isozymes was increased until 7 DAS in pears and subsequently, decreased during storage period. Itai *et al.* (2015) opined that higher activity of acid invertase from 6 to 12 DAS considerably declines sucrose content in Japanese pears. Acid invertase has the highest level during initial storage period in pear fruits (Itai and Tanahashi, 2008). A similar trend of NI activity was observed by Ren *et al*. (2020). The decline in sucrose content until 12 days of ambient storage (Fig. 3D) might be due to conversion into free sugars by various enzymes including SS and invertases (Itai and Tanahashi, 2008). These enzymes also exhibited similar trends in both the cultivars and high temperature improved their activities as shown in fruits of loquat (Wei *et al*., 2017). SPS synthesizes sucrose-6-phosphate molecule which results in the conversion to sucrose by sucrose-6-phophate phosphatase enzyme. Invertase enzymes cleave sucrose into glucose and fructose content. A positive correlation between SS and sucrose content in 'PN' cultivar

suggests that sucrose is synthesized during storage; whereas, a significant negative correlation between sucrose and SS, SPS and invertases depicts sucrose cleavage in 'PB' cultivar.

Cell wall degrading enzymes play an important role in fruit ripening. PME does not have pronounced effect on deviation in the texture of ripening fruit and partial demethylation of pectin occurs before PG causes significant hydrolysis. Thus, PME may function to prepare the substrate for hydrolysis by PG (Awad and Young, 1979). PG catalysis the hydrolysis of (1→4) galacturonan linkages of demethylated pectin and releases shorter chains, thereby causing the depolymerization and dissolution of pectin (Singh and Dwivedi, 2008), cell wall dissolution, and ultimately, fruit softening (Brummell *et al*., 2004). Cellulase acts on cell wall components such as cellulose and xyloglucan of hemicelluloses (Chen *et al*., 2015). In the present studies, the degradation of soluble pectin is related to the higher PG activity in the fruits during ambient storage resulting in softening of flesh. Zhou *et al*. (2011) also observed that a reduction in pectin content in pear fruits during storage might be due to higher depolymerization of cell wall polysaccharides and conversion of pectin's to non-soluble form. Correlation studies revealed a negative relationship between fruit firmness, cellulase and PG enzymes in 'PN' and 'PB' cultivars. The activity of cellulase and PG enzymes increased in both the cultivars which causes decrease in fruit firmness with PG as main enzyme contributing to the degradation of cell-wall polysaccharides. This relation revealed that the cell wall polysaccharides in pear were associated with the fruit softening.

In fruits, optimal concentration of N and K allows a proper development of peel color, fruit size, firmness, TSS, acidity, juiciness, flavor, and aroma. High N content reduces the fruit storability and K is also an important nutrient during storage of fruits to maintain K: Ca ratio (Brunetto *et al*., 2015). Lepaja *et al*. (2018) reported that 'Williams' pear fruit contains 7.83 mg kg^{-1} P, 152.67 mg kg^{-1} K, 11.33 mg kg^{-1} Mg, 10.60 mg kg^{-1} Ca, 1.11 mg kg^{-1} Fe, 1.17 mg kg^{-1} Zn and 1.14 mg kg^{-1} Cu during storage. The concentration of N 3.7 g kg^{-1}, P 1.0 g kg^{-1}, K 10.3 g kg^{-1}, Mg 0.4 g kg^{-1}, Fe 15 mg kg^{-1}, Mn 3.2 mg kg^{-1}, Zn 8.9 mg kg^{-1} and Cu 6.1 mg kg^{-1} was recorded in 'Rocha' pear fruit after storage for 22 days (Saquet *et al*., 2019).

Principal component analysis (PCA) is a multivariate technique to analyze the observations which are described by inter-correlated variables. The sugars are clustered together in one group indicating positive correlations with each other and juice acidity, SS and PG enzymes in second group had positive relationships but both groups had exhibited negative correlations during storage. Similar findings have been reported in pome fruits (Billy *et al*., 2008; Linda-Garcia *et al*., 2019; Li *et al*., 2019).

This study represents the shelf-life of fruits of pear cultivars 'Patharnakh' and 'Punjab Beauty' during storage under ambient conditions. The results showed loss in weight, firmness, pectin and sugar content in fruits of both the cultivars. The activities of cellulase, PG and PME showed the positive effect on fruit softening; hence spoilage occurred during storage of fruits. It can be summarized from the results that reduction in sugar content and fastening of activities of cell-wall degrading enzymes between 6-9 days after storage in 'Patharnakh' and 3-6 days in 'Punjab Beauty' fruits makes them less desirable for further storage under ambient temperature conditions.

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Adv. Hort. Sci., 2021 35(3): 305-318 DOI: 10.36253/ahsc-10630

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Comparison of wild and domesticated hot peppers fruit: volatile emissions, pungency and protein profiles

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Citation:
COMPARINI D., TAITI C., LANZA M., VITA F., PANDOLFI C., LUTI S., SPINELLI F., PAZZAGLI L., MANCUSO S., 2021 - *Comparison of wild and domesticated hot peppers fruit: volatile emissions, pungency and protein profiles.* - Adv. Hort. Sci., 35(3): 305-318.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 9 March 2021
Accepted for publication 3 August 2021

Key words: Artificial Neural Network, *Capsicum spp*. classification, capsaicin, SDS-PAGE, VOCs.

Abstract: *Capsicum* plant species are globally cultivated in warm and temperate regions, being important for agro-economic, biological and cultural aspects. While their worldwide spread and their ability of cross-pollination to easily hybridize play an important role in the formation of numerous species and varieties but also create confusion for their classification. For this reason, the categorization of species and varieties is complex and several methods have been used to evaluate pepper plant origin and evolution. Therefore, the objectives of this study were to compare a wild pepper (*Capsicum chacoense*) with other two domesticated cultivars belonging to different species such as *Capsicum annuum* and *C. baccatum* and draw conclusions about their origins using different approaches. For this purpose three methodologies have been used and compared: the comparison of their fruits volatile organic compounds (VOCs) emissions , their capsaicin and dihydrocapsaicin content and the leaves proteomic profiles. The VOCs analysis has been conducted by a time-of-flight mass spectrometry (ToF-MS) with an innovative approach to better identify all the compounds detected, in particular using two different ionization agents (H_3O^+ and NO^+) to better identify all the compounds detected. The VOCs and pungency analyses were then used to build back propagation neural networks (BPNN) and a Random Tree classifier to conduct a multivariate analysis and evaluate the most species-specific volatiles. The outcomes appeared to be a most accurate approach with respect to the traditional varieties descriptors used for peppers discrimination. The BPNN led to the identification of several putative volatiles as good candidates for the recognition of these species or significant nodes in a decision learning tool. Finally, protein profiles have been obtained by SDS-PAGE analysis on the leaves to perform a fast proteomic comparison among the species. The protein profiles showed the *C. baccatum* and *C. chacoense* were more similar to the domesticated pepper *C. annuum*.

1. Introduction

Origin and classification of Capsicum

The origin of chili peppers has been located in several locations of Latin America as testified by archeological records and pepper is usually classified as one of the first new world domesticated plants (Pickersgill, 1969; Long-Solis, 1986; Perry *et al*., 2007). Peppers represent one of the most ancient plants cultivated in America together with the *Phaseolus* L., maize and other plants of the Cucurbitaceae family, fundamental ingredients of natives' diet. About 36 different species belong to the genus Capsicum and even today there are many wild species to be defined from the taxonomic point of view, consequently, we cannot exclude the existence of new individuals currently unknown (Davenport, 2004). The identification and maintenance of the genetic diversity in *Capsicum* are important to avoid genetic erosion.

Several species exist and *Capsicum* species have recently been described from Bolivia (Nee *et al*., 2006). Among these, only five species respectively C. *annuum* (variety *annuum)*, C. *chinense*, C. *frutescens*, C. *baccatum* (variety *pendolum* and *umbelicatum)* and C. *pubescens* have been domesticated in the past by the American natives and later, in the post-Colombian period, they have been widely imported and cultivated in temperate and tropical regions for the characteristics of their fruits (McLeod *et al*., 1979; McLeod *et al*., 1983). The broad geographical distribution of this genus, usually used as feeds not only by humans but also by birds that don't have receptors for capsaicin, associated with the antiquity of the origin and the high frequency of hybridization, created a broad genetic variability during the evolution and resulted in many morpho-qualitative differences among cultivars of homologous species (Pozzobon *et al*., 2006). In fact peppers plants have been used for several reasons, starting from their high nutritional value, good content in vitamins but also as medicine or mystic rituals. This determined a rapid diffusion of these plants in the old continent, stimulated also by their spicy flavour similar to the black pepper, a very valued spice, whilst peppers presented higher production and more flexibility as food. Furthermore the mechanism of cross-pollination and the ability of peppers to easy hybridize played another role in the formation of numerous varieties with specific features. During the last centuries, botanists have been active to cross-pollinate creating confusion for the classification of the varieties and the identification of indigenes species. For these reasons the zone where each species originated is still subjected to debate. Brazil is considered as the center of origin of the genus capsicum and currently represents the most important source of genetic diversity (Buso *et al*., 2001). For example, a recent survey of chili cultivars from the state of Roraima in northwestern Brazil noted 60 distinct landraces of peppers from four different species: *Capsicum annuum; C. frutescens, C. baccatum and C. chinense* (Barbosa *et al*., 2006). Therefore Brazil and Bolivia possess the highest number of wild species (Pickersgill, 1984), however, a broad and complete study about diversity has not been done yet for the native species. It is anticipated that continuing, plant exploration in southern Peru, Bolivia and Brazil, will yield additional new narrow endemic pepper species (Russo, 2012).

As consequence, a taxonomic classification of peppers and the determination of all relations among species are challenging and created many debates about the origin and evolution of the *Capsicum genus*. Most current peppers are derived from domesticated species. The difficulties in identifying them lie in dialectal names linked with the local tradition, making challenging their classification. For example, a study among the Mexican peppers population underlined that among about 200 common names used to refer to different peppers, only approximately 15 types were different commercial pods (Russo, 2012). This situation contributes in the last filthy years several authors to focus on the taxonomy and origin of the species of this genus without coming out with a complete and flawless analysis (Heiser and Smith, 1953; Pickersgill, 1988; Hunziker 2001; Barboza and Bianchetti, 2005).

It has been proposed that the *C. chacoense* in Bolivia is one of the most ancestral nuclear centers for the origin of *Capsicum* (2n=24) and it is considered as the basal for the evolution of the species (McLeod *et al*., 1982). Furthermore, a study based on karyotype and other considerations of different *Capsicum* by Moscone *et al*. (2007) concluded the existence of a triple origin of domesticated *Capsicum* species. The whole genus has been hypothesized to be originated in the tropical regions of the Americas and the ancestral species should be born in the Chaco zone which extends into several parts in the country of Argentina, Bolivia, Brasil and Paraguay and propose that the genus "chacoense" as one of the most primitive origi-

nated pepper taxon. All the lines that we know today derived from this first line of evolution and have developed and differentiated according to the characteristics of the new habitat: in the north of the Amazon to the ancient forms of *C. annuum,* on the north coast of Brazil and Venezuela in the forms of *C. chinense* and *C. frutescens*; and in a subsequent period from the same ancestral form were differentiated to *C. rhomboideum* (Colombia, Ecuador) and *C. galapagoense* (Galapagos Islands) (Moscone *et al*., 2007).

Starting from a second evolutionary line instead, they are originated different species: in subtropical areas of Brazil and later the *C. baccatum* and *C. eximium*; in the arid regions of Peru the *C. cardenasii, C. tovarii* and *C. pubescens*, while in Paraguay *C. flexuosum* and *C. praetermissum*. Finally, it was hypothesized that the migration of ancestral forms of *C. flexuosum* and *C. praetermissum* gave rise, in some areas of Brazil, the largest center of diversification of the genus represented by the large group of wild species in 26 chromosomes (Moscone *et al*., 2007). Currently, the species that best reflects the ancestral morphologically and physiologically, is the *C. chacoense* (Hunziker, 2001). *C. chacoense* has also been used in breeding *C. annuum* programs, focusing on tobacco mosaic virus resistance (Boukema, 1982). The wild species with 24 and 26 chromosomes present different morphological traits and geographical distribution (Pozzobon *et al*., 2006). The differences that arise are probably related to the different agents of seed dispersal, birds for the first whilst bats and other small mammals for species of 26 chromosomes that present hanging fruit, inconspicuous, and a little spicy. In a second time, the domestication and subsequent human selection caused a selective pressure in favor of large hanging fruits, less attractive for birds with small exception (e.g. *C. frutescens* "Tabasco" varieties), (AISPES, 2010). The classification of chilies, like that of any multifarious group of cultivars, is confusing. For example, a very large amount of pod types exist in *C. annuum*, *C. chinense* and *C. baccatum* (Bosland and Votava, 2012).

Usually, the classification of the genus *Capsicum* and the varieties, belonging to each species, is carried out based on morphological descriptors that define the shape of flowers and fruits (Pickersgill, 1971; Moscone *et al*., 2007; Ince *et al*., 2009; Sudré *et al*., 2010) and the current system for classifications involved genus, species, variety, pod type and cultivars (Bosland and Votava, 2012). However, other descriptors are considered essential for an accurate germplasm characterization, such as those indicated by IPGRI (International Plant Genetic Resources Institute). Furthermore, the characterization and evaluation of the species belonging to the genus *Capsicum* are particularly interesting for breeders, gene banks, because of the large genetic variability available (Guzmán *et al*., 2005; Sudré *et al*., 2006; Ince *et al*., 2009). They can furthermore be identified from the different flavors of the fruits and two main factors that contribute to the aroma perception are pungency and aroma, and these are associated with the fruit volatile compounds (Taiti *et al*., 2015).

With the advancement of computer technology, multivariate methods have become an important tool for taxonomic classification (Ortiz *et al*., 2008). However, the procedures of statistical classification require a data set based on a large number of variables. Thus, the paper has aimed to analyze and characterize the wild *C. chacoense* and to compare its profile of volatile emissions, pungency, and proteins with two domesticated species i.e. *C. annuum* and *C. baccatum* to elucidate the evolution of the *Capsicum* genus. For this reason, the volatiles compounds emissions profile of each species has been used to build an Artificial Neural network and differentiate and identify the species. Finally, an additional proteomic analysis of the leaves of the peppers has been used to evaluate the relationships among the species and compare the results obtained from the volatiles emission, pungency and protein expression profiles of each species.

2. Materials and Methods

Fresh pepper material

Ripe pepper fruits belonging to 3 different species were used in this study: *Capsicum annuum* var. Ciliegino, *C. baccatum* var. Brasileiro and *C. chacoense* (wild accession). The fruits were collected from ten plants, for each species, grown into greenhouse (Florence, Italy). Fully matured uniform-sized fruits were collected within 24h after the 100% color surface was reached. All plants were obtained from seeds, germination and growing phases were made following the same system used in the previous work of Taiti *et al*., 2015. All plants belonging to each species were grown in the greenhouse in three different rooms to avoid the effects of cross-pollination.

Capsaicin and dihydrocapsaicin quantification

Capsaicinoids are a group of alkaloids produced as

secondary metabolites by chili peppers responsible for the pungency. Among several structural analogs capsaicinoids, capsaicin and dihydrocapsaicin are the two most potent and abundant compounds accounting for more than 90% of total capsaicinoids in chili pepper (Ziino *et al*., 2009). Capsaicin and dihydrocapsaicin have been extracted from whole frozen fruits and calculated as the average of five extraction for each species (n=5, SD). About 2 g of frozen fruits have been weighted and then pulverized in 10 mL of cold acetone at 4°C and kept overnight. Then 50 µl of the sample have been collected by using a 0.22 µm filter syringe and used for the quantification. All data have been calculated as µg of capsaicin or dihydrocapsaicin content per gram of fresh weight after normalization based on the exact weight of the initial fresh sample.

RP-HPLC quantification of capsaicin and dihydrocapsaicin was performed by using a C18 column, 3 µm, 15x4.6 cm (Supelco, Bellefonte, Pennsylvania, USA). The stock solution containing capsaicin and dihydrocapsaicin (cod, 360376 Sigma-Aldrich, St Louis, MO, USA) was prepared in 20% Acetonitrile at concentrations of 7.5 mg/mL. For calibration curves construction 30 µl, 60 µl, 90 µl, 120 µl, 150 µl, 300 µl and 500µl of stock solution were analyzed obtaining a linear curve for both capsaicin and dihydrocapsaicin (R^2=0.9912 and R^2=0.9923, respectively). Elution gradient was performed at a flow rate of 0.8 ml min^{-1} with the following solvent system: 10mM trifluoroacetic acid (TFA) in acetonitrile (solvent A); 10mM TFA in water (solvent B). The gradient used was 20% A for 2 min, from 20% to 100% A in 15 min, holding at 100% A for 10 min, from 100% A to 20% A in 2 min, and detection was based on UV absorbance at 280 nm. Under these conditions, the capsaicin peak appeared at a retention time (Rt) of 10.6 min and dihydrocapsaicin at Rt of 11.3 min. Quantification was calculated using the Chromeleon software.

Volatiles organic compounds (VOCs) analysis

SRI-MS ToF protocols. For headspace analysis, pepper fruits have been selected among ten plants and the uniform-sized fruits were collected at the optimal ripening stage (100% of coloration). For each species ten samples were analyzed, each constituted of 10 grams of fresh pepper fruits. Using a commercial PTR-TOF 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) instrument with SRI-MS (Switchable Reagent Ions - Mass Spectrum) upgrade, the analysis of the samples was carried out following a similar procedure described in Taiti *et al*. (2015). In short, 10 g of freshly cut chili pepper (including the seeds) without any pre-treatment were placed in a glass jar (750 mL at 25°C, with a dynamic headspace flushing flow rate of 200 mL min^{-1}) equipped with two Teflon inlet and outlet tubes on the opposite side, which were respectively connected to a zero-air generator (Peak Scientific) and the PTR-TOF MS instrument. Moreover, for the first time, the Switchable Reagent Ion System (H_3O^+ and NO^+) has been used to produce different ionization agents for capsicum study. Using the additional precursor (reagent) ions as NO^+, besides the usual H_3O^+, improved the analytical possibilities of this technique (Wang *et al*., 2004; Mochalski *et al*., 2014). In particular, the SRI system allows: (1) the analysis of VOCs which are not detectable with the reference ion H_3O^+ (e.g. alkanes); (2) the separation of isobaric compounds as in the case of aldehydes and ketones using NO^+ (Jordan *et al*., 2009; Del Pulgar *et al*., 2013). For a detailed explanation of the system see Blake *et al*. (2006) and Blake *et al.* (2009). All samples were analyzed using the same procedure and the VOCs were assessed with H_3O^+ and NO^+ as reagent ions; the drift conditions for each primary ion used are reported in Table 1. The sampling time for each channel of TOF acquisition was 0.2 ns, for a mass spectrum comprised between *m/z* 20-210. The duration of a single sample measurement was 120 seconds, which corresponds to 60 mass spectra. All the samples were analyzed in an air-conditioned room, with a constant temperature of 25±1°C (Mancuso *et al*., 2015). The SRI-MS upgrade consists of an additional mass flow controllers for the respective reagent gases (water vapor for H_3O^+, charcoal filtered air for NO^+); for a detailed explanation see Jordan *et al*. (2009). In short, the use of NO^+ and the H_3O^+ as a reagent ion

Table 1 - Instrumental condition throughout the experiment

Primary Ion	Drift voltage (V)	Pressure (mB)	Temperature (°C)	Us (V)	Uso (V)	Ihc (mA)	Udx (V)	E/N (Td)	Mass calibration	Mass calibration	Mass calibration
H_3O^+	594	2.25	110	110	85	4.0	35	140	21.022	29.997	59.041
NO^+	600	2.30	110	25	80	5.0	36	137	21.022	32.002	47.997

improves the analytical performance of the tool, particularly for the separation of isobaric compounds and for the detection of compounds with proton affinities lower than that of water. Furthermore, as reported by Edtbauer *et al.* (2014) when the PTR-MS instrument works in NO^+ mode can improve the selectivity of compounds detection.

Mass data statistical analysis. Since the external calibration provided by the tool gave a poor mass accuracy, it has been performed off-line thus ensuring, a high mass accuracy generally than 0.001Th, which in most cases allowed the formula identification (Taiti *et al*., 2017). The raw data of each peak spectrum (calculated as number of counts per second, cps) were acquired with the software TOFDaq (TOFwerk AG, Switzerland) by setting a dead time of 20 ns for the Poisson correction, instead, for peak quantification, the resulting data were corrected according to the duty cycle and the signals were normalized (ncps "normalized count per second") as described by Herbig *et al*. (2009). Moreover, the Poisson correction has been applied to correct all spectra for any count losses. Finally, VOCs putative identification was based on a high instrumental mass resolution and the fragmentation patterns of pure standards available in the bibliography (Buhr *et al*., 2002; Lee *et al*., 2006; Maleknia *et al*., 2007; Kim *et al*., 2009; Tani, 2013; Aprea *et al.*, 2015; Taiti *et al.*, 2019) and integrated with previously detected VOCs emitted from *Capsicum* fruit available in literature (Table 2 and 3).

Data processing and classification methods

Back propagation neural network. In this study, the capsaicinoid contents and the 52 volatile signals detected by the PTR-ToF-MS by using H_3O^+ as reagent ion were used as input layers, and the 3 pepper species represented the output (30 single pepper fruits analysis, ten for each species). The BPNN was built using a data mining software (Weka 3.6.14) and the Multilayer Perceptron classifier was used for the classification.

Two BPNN were made, one with only the VOCs emission profiles, and another with VOCs and capsaicinoids. The number of hidden neurons and the number of iterations was adjusted to optimize the neural network activity. Many factors, such as learning schemes, numbers of nodes, and connections between them, play an important role in determing of the best configuration of the hidden layers (Zurada

Table 2 - List of the average m/z-signals that can be specifically assigned using H_3O^+ as reagent ion: Volatile Organic Compounds headspace intensity expressed in ncps (n=10; ±SD); chemical formulae and tentative identifications for each signals detected; the compounds identification was linked to the PTR-ToF-MS pattern fragmentation references [(a)] or previously reported in *Capsicum* species [(b)]

	Measured mass (*m/z*)	*Capsicum Annuum* Ciliegino	*Capsicum Baccatum* Brasileiro	*Capsicum Chacoense* Wild pepper	Sum formula	Tentative identification	PTR Pattern fragmentation [a]	Capsicum Literature [b]
1	27.022	799.86±234.50	200.93±109.28	496.95±140.33	$C_2H_3^+$	Acetylene		
2	31.018*	615.25±205.40	184.12±62.55	81.22±21.30	CH_3O^+	Formaldehyde	[2]	
3	33.033	1712.59±220.12	415.39±167.27	1067.70±200.55	CH_5O^+	Methanol	[1]	
4	41.038	2612.818±638.34	983.12±320.11	378.78±100.33	$C_3H_5^+$	Alkyl fragment (Alcohols and esters)	[1]	
5	43.054	578.30±190.22	133.23±33.90	101.94±25.90	$C_3H_7^+$	Alkyl fragment (Alcohols)	[1]	
6	45.033	3049.50±982.28	529.04±67.40	5638.53±1230.45	$C_2H_5O^+$	Acetaldheyde	[2]	
7	47.010	353.83±111.89	224.89±51.58	636.43±160.55	$CH_3O_2^+$	Formic Acid/Formates	[2]	
8	47.049	4.80±1.20	3.50±0.80	2.95±1.00	$C_2H_7O^+$	Ethanol	[1]	Rodriguez-Burruezzo *et al*., 2010
9	53.030	102.26±35.40	50.90±22.33	17.53±5.00	$C_4H_5^+$	Cyclobutadiene		
10	55.050	6.82±1.90	1.99±0.50	3.32±1.30	$C_4H_7^+$	C4 aldehydes fragment	[1]	
11	57.033*	2910.50±1109.12	366.28±78.54	179.55±49.50	$C_3H_5O^+$	C3 aldehydes and ketones fragments	[6]	
12	57.069	274.43±85.40	45.91±17.22	33.02±9.25	$C_4H_9^+$	Alcohol fragments (1-Butanol, 1-Pentanol, 1-Hexanol, 2-Methyl-1-propanol, Pentanol, 1-Heptanol, Octanol, Nonanol)	[1]	
13	59.049*	553.06±133.09	196.04±65.90	459.80±180.44	$C_3H_7O^+$	Propanal, Acetone	[2]	Ziino *et al.*, 2009
14	61.028	303.46±44.65	120.22±39.10	355.05±44.44	$C_2H_5O_2^+$	Acetates	[6]	Ziino *et al*., 2009
15	63.027	4.45±1.30	4.64±1.20	5.05±0.80	$C_2H_7S^+$	Dimethylsulfide		Taiti *et al*., 2015

* The signals that mostly contributed to the BPNN classification have been marked.

to be continued...

Table 2 - List of the average m/z-signals that can be specifically assigned using H_3O^+ as reagent ion: Volatile Organic Compounds headspace intensity expressed in ncps (n=10; ±SD); chemical formulae and tentative identifications for each signals detected; the compounds identification was linked to the PTR-ToF-MS pattern fragmentation references [(a)] or previously reported in *Capsicum* species [(b)]

	Measured mass (*m/z*)	*Capsicum Annuum* Ciliegino	*Capsicum Baccatum* Brasileiro	*Capsicum Chacoense* Wild pepper	Sum Formula	Tentative identification	PTR Pattern fragmentation [a]	Capsicum Literature [b]
16	65.038	4.87±3.25	2.49±0.80	3.45±1.11	$C_5H_5^+$	Alkyl fragment/ Terpenes fragment	[1/2]	
17	67.050	61.35±16.66	13.86±6.40	6.77±1.40	$C_5H_7^+$	Terpenes fragment	[2]	
18	69.033	80.90±21.98	17.82±4.50	11.92±4.10	$C_4H_5O^+$	Furan		
19	69.069	395.06±133.90	139.11±80.33	92.50±18.44	$C_5H_9^+$	Isoprene/Alkyl fragment (e.g. 2-methylbutanal, 1-octen-3-ol)	[2/6]	
20	71.049*	21.29±7.10	6.52±2.12	8.70±2.50	$C_4H_7O^+$	2-Butenal		Taiti *et al.*, 2015
21	71.086	15.92±5.90	4.71±190	7.65±1.90	$C_5H_{11}^+$	Alchol (3-methyl-1-butanol, Pentanol, Iso-pentanol, 2-ethyl-1-hexanol,		Eggink *et al.*, 2012 a
22	73.060*	43.02±12.35	6.62±2.10	15.45±4.90	$C_4H_9O^+$	Isobutanal/Butanone/Butanal	[1]	Ziino *et al.*, 2009
23	75.044	57.76±22.25	14.33±4.50	12.16±6.00	$C_3H_7O_2^+$	Butanol/Methyl acetate/Propanoates	[1]	
24	77.038	7.09±2.79	7.77±2.20	14.53±3.50	$C_6H_5^+$	Alkyl fragment		
25	79.054	134.86±48.60	22.84±7.98	15.7±4.10	$C_6H_7^+$	Benzene/Alkyl and terpenes fragment	[2]	
26	81.068	949.61±301.39	178.02±68.78	152.06±50.30	$C_6H_9^+$	Terpenes fragment/Aldehydes fragment (trans- 2-hexenal)	[4]	Taiti *et al.*, 2015
27	83.049	76.90±19.75	32.36±9.94	13.69±2.90	$C_5H_7O^+$	2-Methylfuran		Rodriguez-Burruezzo *et al.*, 2010
28	83.086	175.59±50.10	92.78±29.50	16.29±4.90	$C_6H_{11}^+$	C6 compounds/ Hexenol fragment	[6]	
29	85.064	46.91±15.40	9.90±3.10	18.20±4.30	$C_5H_9O^+$	Methyl-butenal/1-penten-3-one		Ziino *et al.*, 2009
30	85.101*	32.25±12.30	2.08±0.94	7.41±2.00	$C_6H_{13}^+$	Alcohol (1-Hexanol/Nonanol)		Ziino *et al.*, 2009
31	87.045*	25.21±5.40	6.92±3.20	12.65±4.10	$C_4H_7O_2^+$	Diacetyl /2,3-butanedione	[1]	Ziino *et al.*, 2009
32	87.080	10.23±1.21	6.44±0.95	2.01±0.55	$C_5H_{11}O^+$	2,3-Methylbutanal /(Z)-2-penten-1-ol/3-Pentanone	[1]	Rodriguez-Burruezzo *et al.*, 2010
33	91.075	22.38±5.40	17.68±4.40	6.35±1.10	$C_4H_{11}O_2^+$	2,3-Butanediol/Monoterpene ketone	[5]	Ziino *et al.*, 2009
34	93.069	7.07±1.95	12.12±5.40	7.59±1.50	$C_7H_9^+$	Terpene fragments (e.g. cymene, limonene)	[3]	Rodriguez-Burruezzo *et al.*, 2010
35	95.086	27.45±6.99	4.18±2.20	14.97±1.10	$C_7H_{11}^+$	1-Methyl-1,4-cyclohexadiene		Eggink *et al.*, 2012 b
36	97.064	50.45±10.50	14.90±4.80	10.18±2.50	$C_6H_9O^+$	2-Ethylfuran		Rodriguez-Burruezzo *et al.*, 2010
37	99.080*	60.65±20.30	8.62±2.75	15.98±6.30	$C_6H_{11}O^+$	cis-3-Hexenal/ (E)-2-Hexenal		Rodriguez-Burruezzo *et al.*, 2010
38	101.096	33.66±5.70	9.62±3.55	17.02±5.50	$C_6H_{12}O^+$	Hexanal/ (E)-2-Hexenol	[1]	Ziino *et al.*, 2009
39	103.075	54.22±20.44	18.20±6.50	9.28±2.80	$C_5H_{11}O_2^+$	3-Methylbutanoic acid	[6]	Zimmermann and Schieberle, 2000, Azcarate *et al.*, 2010
40	105.069	4.98±0.65	5.23±0.78	5.96±1.12	$C_8H_9^+$	Styrene/Styrol/Phenylethanol	[6]	Rodriguez-Burruezzo *et al.*, 2010
41	107.085	9.50±5.44	3.65±2.30	12.85±7.20	$C_8H_{11}^+$	p-Xilene	[6]	Eggink *et al.*, 2012 a
42	109.101	13.85±4.50	3.15±0.90	6.06±2.20	$C_8H_{13}^+$	Terpenes fragments	[3]	
43	115.111*	17.27±4.30	6.04±2.50	1.36±0.50	$C_7H_{14}O^+$	Heptanal	[6]	Ziino *et al.*, 2009
44	117.091	6.35±1.60	14.05±3.00	3.96±1.30	$C_6H_{13}O_2^+$	Hexanoic acid/Hexanoates	[6]	Eggink *et al.*, 2012 b
45	119.085	7.75±3.70	3.26±1.35	4.89±2.40	$C_9H_{11}^+$	Terpenes fragment	[3]	Ziino *et al.*, 2009
46	121.101	6.40±2.50	15.08±3.50	6.10±2.00	$C_9H_{13}^+$	Terpenes fragment	[3]	
47	123.120	8.40±2.80	1.65±0.60	1.72±0.85	$C_9H_{15}^+$	Sesquiterpene fragments	[4]	
48	135.117*	7.15±1.83	2.69±1.40	6.62±1.30	$C_{10}H_{15}^+$	p-Cymene/Monoterpene ketone fragment	[6/5]	Ziino *et al.*, 2009
49	137.132*	17.12±4.05	9.75±1.10	23.88±4.35	$C_{10}H_{17}^+$	Monoterpenes (e.g. (Z)-b-ocimene)	[5]	Eggink *et al.*, 2012 a
50	149.132*	7.13±4.40	3.91±1.05	9.65±3.20	$C_{11}H_{17}^+$	Sesquiterpenes fragments (e.g. Ectocarpene)	[4]	Taiti *et al.*, 2015
51	205.195	25.44±7.10	11.90±3.33	9.59±2.65	C15H25+	Sesquiterpenes	[4]	Eggink *et al.*, 2012 b
TOTAL VOCs EMISSION (average ncps)		22116	4850	13214				

* The signals that mostly contributed to the BPNN classification have been marked.

Table 3 - SIFT-MS signals obtained using NO+ as reagent ions, in the range between to m/z 20-200. The analysis showed only the signals with intensity expressed in ncps higher than 1 (n=10; ±SD)

Number of compounds	Measured mass (m/z)	*Capsicum Annuum* Ciliegino	*Capsicum Baccatum* Brasileiro	*Capsicum Chacoense* Wild pepper	Sum Formula	Tentative identification
1	41	10.91±3.10	45.53±16.60	19.47±8.85	$C_3H_5^+$	Alkyl fragment (Alcohols and esters)
2	45	18.72±2.10	25.67±8.40	21.61±6.80	$C_2H_5O^+$	C6 fragment (e.g. (E)-2-Hexenol)
3	57	7.41±2.98	48.61±18.07	9.68±1.55	$C_3H_5O^+$	C3 Aldehyde
4	58	50.01±8.86	39.10±3.25	25.63±9.52	$C_3H_6O^+$	C3 ketones
5	69	13.07±2.33	45.73±18.80	20.95±8.55	$C_4H_4O^+$	Furan
6	83	15.11±2.23	79.05±20.44	13.85±0.60	$C_6H_{11}^+$	C6 aldehydes (e.g (E)-2-Hexenol)
7	84	4.17±0.88	3.64±0.55	2.94±0.90	$C_5H_8O^+$	C5
8	85	7.62±3.20	27.85±7.22	10.69±3.33	$C_5H_{11}O^+$	Valeraldehyde
9	88	59.06±24.44	44.29±27.70	61.12±20.32	$C_3H_6O\ NO^+$	Acetone
10	99	11.12±2.33	15.24±3.33	10.76±2.22	$C_6H_{13}O^+$	Hexanal/ C6 aldehydes (e.g (E)-2-Hexenol)
11	106	3.67±0.78	2.87±0.30	2.780±6.52	$C_8H_{11}^+$	Xylene
12	113	1.05±0.33	6.65±.55	1.34±0.55	$C_7H_{13}O^+$	Heptenal
13	114	2.60±0.44	10.15±1.1	2.16±0.30	$C_5H_8NO_2^+$	Cluster C5 unsaturated ketones
14	116	9.47±2.63	26.33±8.33	6.39±1.20	$C_5H_{10}NO_2$	Cluster C5 ketones/Pentanone
15	128	3.34±0.81	2.04±1.01	1.88±0.72	$C_8H_{16}O$ +	6-Methyl-5-hepten-2-ol
16	136	17.85±1.66	7.78±0.95	26.57±6.77	$C_{10}H_{16}^+$	Monoterpene compounds
17	144	0.80±0.62	3.21±0.81	0.70±0.55	$C_6H_{10}NO_3^+$	Cluster hexanedione/heptanone
18	166	0.73±±55	2.38±0.35	0.80±0.68	$C_{10}H_{16}NO^+$	Monoterpenes fragment
Total VOCs emission average (ncps)		236.71	436.09	239.29		

1992; Zurada and Malinowski, 1994). In our case, the minimum error was reached with a network composed of 29 hidden neurons for both BPNN, positioned on one level, with the hidden layer activated by a logistic sigmoid activation function:

$$f(x) = 1/(1+e^{-x}) \quad (1)$$

These sigmoid functions fix the output signal limit between 0 and 1. The resulting function works as an output logic-gate that can be opened (1) or closed (0). Also, as part of a continuous function it can happen that a gate is partially opened (i.e., its value results between 0 and 1). Ideally, only a group of outputs, which represents an accession, would express a value of 1 (meaning correct identification) while the remaining groups would show a value of 0 (incorrect identification). In reality, this take place rarely, for this reason it is usually considered as "incorrect" a value closer to zero (wrong identification), while "correct" when the resulted value is close to 1 (correct identification) (Pandolfi *et al*., 2009).

A 10-folds cross-validation was applied to test the performance of the model. The original dataset was essentially randomly segregated into 10 equal-sized groups. Each set is divided into two groups: 90% of data are used for training the network and 10% of data are used for the validation test. The cross-validation process is then repeated 10 times (the folds), in which every time a subsamples is validated. The results deriving from all folds are finally averaged to result in a single evaluation of the network's performance.

The values of the identification for each species were highlighted using a misidentification matrix. All identification processes executed by the network were averaged and the results represented in Table 4. The rows refer to the species in the test set, the columns report the species to which the test plants are referred by the neural network. An "Attribute selection filter" provided by Weka was also applied to the two sets of data, to determine the more discriminant parameters.

Random tree. The 51 volatile signals detected by the PTR-ToF-MS using H_3O^+ (30 single pepper fruits analysis, ten for each species) were also analyzed using the "Random tree" algorithm, a decision tree

Table 4 - Confusion Matrix derived from the Random tree analysis from the aromatic profiles of the pepper species obtained using a decision tree learning tool

	C. baccatum	*C. chacoense*	*C. annuum*
C. baccatum	9	1	0
C. chacoense	0	10	0
C. annuum	0	0	10

learning tool provided by the software (Weka 3.6.14). In classification trees, each internal node is labeled with an input feature, which can be informative to detect similarities or differences among the pepper species.

Similarly to the BPNN, a 10-folds cross validation was applied to test the performance of the model, and the results from the folds are then averaged to produce a single estimation of the performance of the algorithm.

SDS-page protein analysis

Protein extraction and quantification. Soluble proteins were extracted from leaves of three Chili pepper plants of each species (*Capsicum Chacoense, C. annuum* and *C. Baccatum)* according to Vita *et al*. (2013), with some modifications. In short, for each analysis 100 mg of fresh leaves were grounded in liquid nitrogen and homogenized with 1 mL of extraction buffer (5 M urea, 2 M thiourea, 40 mM Tris-HCl, 2% CHAPS, 50 mM DTT). The homogenates were centrifuged for 15 min at 15,000 rpm. Supernatants were precipitated using TCA (15%, v/v) containing 0.007% β-mercaptoethanol in acetone at -20°C for 2 h and successively at 4°C for a minimum of 2 h. Samples were centrifuged at 4°C for 15 min at 14,000 rpm, supernatants were discarded and pellets were washed twice with ice-cold acetone containing 0.007% β-mercaptoethanol. Pellets were dissolved in a rehydration buffer (5 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT). Protein quantification was performed using a Bradford-based assay kit assay (Bio-Rad Hercules, CA), using bovine serum albumin as a standard.

SDS-PAGE. The protein separation was carried out by the established technique of SDS-PAGE. In detail, the gels used (size 20 * 20 cm) had a thickness of 1 mm and were constituted by a stacking gel (4.8% T, 1.3% C, pH 6.8) and a running gel (15%, 1.3% C, pH 8.5). SDS-PAGE analyses were performed 4 times (n=4). Electrophoresis runs were carried out using the Protean XI cells (Bio-Rad Laboratories, Inc, Hercules CA) with specific parameters (for each gel 25 mA, 8h running time, temperature 15°C). Precision Plus Protein™ Unstained was the molecular marker used for the essay (Bio-Rad Laboratories Inc., Hercules, CA). The protein samples were analyzed by SDS-PAGE on gels stained with the Brilliant Blue G-Colloidal Concentrate Coomassie (Sigma-Aldrich) according to the manufacturer's instructions.

The images of each gel were acquired using a Bio-Rad densitometer GS-800 ™ in greyscale colors, with a definition of 300 dpi. The images were analyzed using the software Quantity One 1-D Analysis ™ software (Bio-Rad Laboratories, Inc, Hercules CA). Dendrogram based on signal quantities was created using correlation-based distances and Ward's method of agglomeration was used in the present analysis (Ward, 1963).

3. Results and Discussion

Capsaicinoid

In all the species analyzed both the capsaicin and the dihydrocapsaicin (DHC) contributed to the pungency of the fruits. As expected (Stoica *et al*., 2016), the capsaicin content was higher than the DHC in all species and C. *chacoense* resulted in the higher content of about 300 µg/g of fresh weight (FW) compared to C. *baccatum* whilst C. *annum* have been the most variable samples with an high standard deviation that made it not statistical different from the other two species (Fig. 1). Interestingly the C. *chacoense* resulted in the higher content of DHC of 243±36 µg/g FW, followed by the C. *annum* and C. *baccatum* that did not resulted statistical different

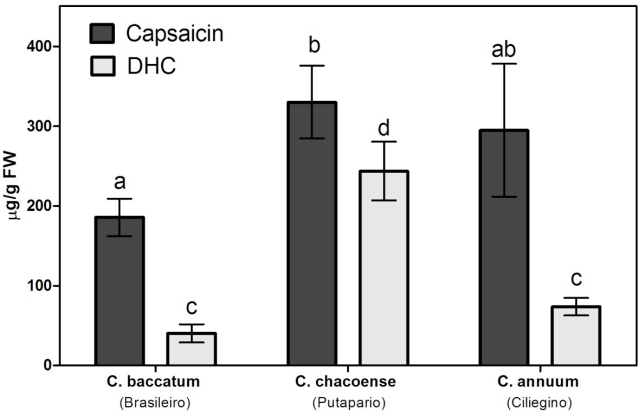

Fig. 1- Capsaicin and dihydrocapsaicin (DHC) content obtained from ripe fresh pepper fruits for each species analyzed, using HPLC detection (n=5; SD). Different letters represent statistical significance (ANOVA, p<0.05).

from each other with respectively 73±11 µg/g FW and 40±11 µg/g FW. The capsaicin content influences more the spiciness followed by the DHC, therefore *C. baccatum* have showed the lowest level of spiciness.

PTR-ToF-MS volatiles compounds analysis

The volatiles compounds analysis by SRI-ToF-MS revealed the volatile profile of each pepper species. The extraction of every single peak permitted the detection and the quantification of specific signals resultant of the protonation of numerous VOCs (*m/z* range = 20-210). From the volatile fraction composition of different pepper species, were detected many volatile compounds specifically alcohols, aldehydes, esters, ketones, hydrocarbons and terpenes compounds. For each peak identified using H_3O^+ and NO^+ as ion reagents, all of the *m/z* detected have been assigned to the mass formulas reported respectively in Table 2 and 3 and expressed in ncps higher than 1 (n=10; ±SD). In particular, by trusting the high accuracy and resolution of this tool, the chemical compounds have been tentatively proposed and matched with the existing documentation of VOCs in literature and by the acknowledged VOCs emitted by peppers. Thus, the identification of the compounds has been further improved by the use of two different reagents ions. All the peaks obtained have been filtered and 51 mass spectral peaks have been detected when H_3O^+ was the reagent ion and 18 using NO^+ as reagent ion.

Remarkably even if the pepper analyzed derived from different species (in particular *Capsicum Chacoense*), all the peaks identified using H_3O^+ or NO^+ as reagent ions were always present in the three species of chili pepper included in this study. Moreover, since the aroma is linked to the species and varieties (Taiti *et al*., 2019) the differences of VOCs emission among these three hot peppers were expected (Table 2 and 3). Above all, by using H_3O^+, *C. annuum* showed the highest total VOCs emission (22,116 ncps) followed by C. *chacoense* (13,214 ncps) and C. *baccatum* (4,850 ncps) as reported in Table 2. In particular, C. *annuum* ("Ciliegino" var.) seems to have a richest volatile profile compared to the other ones and showed the highest VOCs intensity for the compounds linked to the herbaceous notes. This trend could be confirmed by the high intensity of compounds detected at m/z 81.069, 83.086, 85.101, 99.080 and 101.096 all identified as C6 compounds (Table 2). Notably, peaks that confirmed this trend were detected at $C_4H_9^+$ (measured at *m/z*=57.069) probably derived from Alkyl fragment (Hexanol/valeric acid), $C_5H_7^+$ (measured *m/z*=67.055) and $C_6H_9^+$ (measured *m/z*=81.069) likely correspond to Alkyl fragment (isoprene and terpenes or aldehydes fragments respectively), $C_6H_{13}^+$ (measured *m/z*=85.101) probably refers to fragments of 1-Hexanol and/or Nonanol, $C_5H_{11}O_2^+$ (measured *m/z*=103.075) refers as 3-Methylbutanoic acid and $C_{15}H_{25}^+$ (measured *m/z*=205.195) attributed to Sesquiterpene compounds. On the contrary, the VOCs emission observed in the wild accession (*C. chacoense*) is characterized by the high emission of signals detected at m/z 33.033, 45.033 and 137.132, with the first two compounds which are linked to the ripening process whilst the last compound belongs to the terpene class. Moreover, it is interesting to note that the signal intensity of monoterpenes (*m/z* 137.132) is higher in *C. chacoense* than that observed for sesquiterpenes (*m/z* 205.101), in contrast to the emissions of *C. annum* and *C. baccatum* (Fig. 2, Table 2). Regarding the wide chemical classes of terpenes, common compounds which strongly contributes to the aroma of fruits and vegetables (Rodrîguez-Burruezo *et al.*, 2010), in this study were identified several peaks belonging to monoterpenes (C-10) and sesquiterpenes (C-15) while no one was detected as oxygenated terpenes. This result is also confirmed by what was already reported (Rodrîguez-Burruezo *et al*., 2010), where the oxygenated terpenes were found at only traces and mainly in *C. chinense*.

Finally, the aroma of "Brasileiro" variety (C. *baccatum*) was characterized by a lower signals intensity, except for the compounds detected at m/z 117.091 (TI: Hexanoic acid/Hexanoates) and m/z 121.101 (TI: Terpenes fragment). Additional representative chemical classes of the volatile fraction were aliphatic

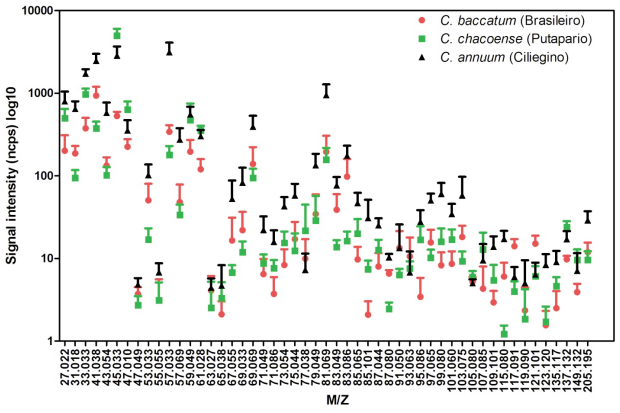

Fig. 2 - Example of schematic chart of mass peaks detected with H_3O^+ ion as reagent for each capsicum species used in this paper. Signal intensities are given in normalize count per second (ncps) log^{-1} and higher than 1.

aldehydes, alcohols, and branched hydrocarbons. In particular, Hexanal, Hexanol, cis-2-Hexanal and cis-2-Hexenol were the main compounds that contribute to the flavour note described as the odour of freshly cut grass or ground leaves, which are typically produced in fresh *Capsicum* fruits later on the tissue destruction (Ziino *et al.*, 2009). On the contrary by using NO^+ as donor ion, the differences of VOCs emission among all varieties studied were smaller as well as the number of peaks detected (Table 3). Sometimes these spikes were different, while sometimes were identical to those obtained using H_3O^+ as a reagent ion and this behavior is usual and has been already reported elsewhere (Jordan *et al*., 2009). Indeed, even if we use NO^+ as reagent ion in a complex matrix, H_3O^+ could occur in the ionization of the compounds present in the sample headspace (Jordan *et al*., 2009, Del Pulgar *et al*., 2013). Interesting, in contrast to what was observed with H_3O^+ , when using NO^+ the Capsicum species *C. baccatum* showed for the majority of signals the highest intensity detected (Table 3). Moreover, Table 3 shows significant differences in the concentration of many peaks between the three pepper species, especially as far as they are concerned with protonated aldehydes and ketones.

Artificial neural networks

Two neural networks have been built: the first one uses only VOCs emission, the second one combines the pungency with capsaicin and DHC analysis as inputs layers. Both the ANN (Artificial Neural Network) were able to discriminate among the accession with 100% accuracy. Thus, the confusion matrix assigns all the aromatic profiles correctly to the related pepper species (Table 4). According to the attribute selection filter applied, 12 VOCs profile and DHC content were the most discriminant parameters. In particular, the *m/z* signal of the VOCs and the tentative identification are reported here below and are marked in Table 2 with the asterisk symbol: *m/z* 31.018 (Formaldehyde), *m/z* 57.033 (C3 aldehydes and ketones), *m/z* 59.049 (Propanal, Acetone), *m/z* 71.049 (2-Butenal), *m/z* 73.060 (Isobutanal/Butanone), *m/z* 85.101 (1-Hexanol/Nonanol), *m/z* 99.080 (cis-3-Hexenal/(E)-2-Hexenal), *m/z* 115.111 (Heptanal), *m/z* 135.117 (p-Cymene), *m/z* 137.132 (Monoterpenes) and *m/z* 149.132 (Sesquiterpenes fragments).

Random tree

The data coming from the aromatic profiles of the pepper species were also analyzed using a decision tree learning tool. The identification was successful in 96.6% of the cases, and, as shown in the confusion matrix, Table 4, only one instance belonging to *C. baccatum* was incorrectly attributed to *C. chacoense.* The decision tree showing the two significant nodes is reported in figure 3. According to the tree, only two VOCs are fundamental to discriminate among the three species: *m/z* 81.068 and *m/z* 85.064.

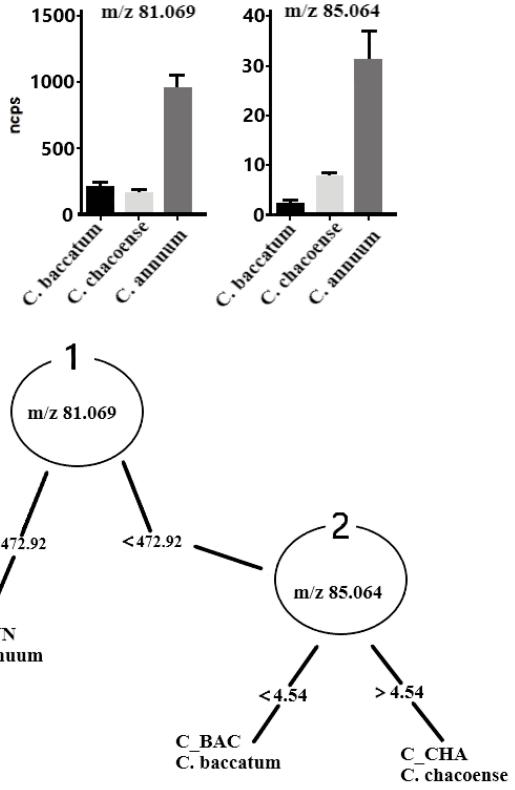

Fig. 3 - On the top the VOCs signal of the two most significant masses that help the identification of the species using a decision tree learning tool (n=10; SD). On the bottom, the two significant nodes have been reported.

Protein profiles

SDS-PAGE analyses performed on *Capsicum* species (Fig. 4) identified specific profiles linked to each sample. Gel images were then analyzed to generate data like a phylogenetic tree based on similarity comparison (Fig. 4) to graphically display relationships among samples. Dendrograms results showed as two samples, *C. chacoense* and *C. baccatum,* clusterized independently from the third sample *C. annuum.* Differences detected in the protein profiles could be associated with quantitative differences in the band densities as soon as some quantitative differ-

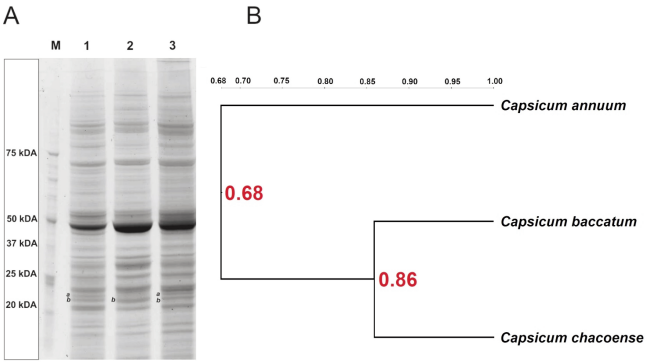

Fig. 4 - (A) SDS-PAGE analysis of proteins getting from *Capsicum* samples, where 1= *C. chacoense*; 2= *C. annuum*; 3= *C. baccatum*; M= molecular marker. (B) Phylogenetic tree resulting from the analysis of protein by SDS-PAGE analysis. Image data were processed with Quantity One software (Bio-Rad Laboratories, Inc, Hercules CA) using Ward's method for clustering (Ward, 1963).

ences. *C. chacoense* and *C. baccatum* showed a band (a) with a molecular weight slightly higher 20 kDa that were not detected in the *C. annuum* sample (Fig. 4).

4. Conclusions

Our study underlined the high potential of the using the PTR- SRI-MS to obtain a species-specific fingerprinting of the volatile compounds emitted by the pepper fruits. This technology can help to highlight particular VOCs signals that are specific of species or specific growing conditions of chili pepper fruits with a rapid analysis without any pre-treatment of the samples. Indeed, the switching reagent ion system in PTR-MS instrumentation was applied for the first time to analyze pepper fruits by using not only H_3O^+ but also NO^+ as precursor ions. This tool has permitted to find the VOCS able to discriminate among the species and by using two ionization agents a more accurate identification of the volatile compounds has been possible. In particular, the PTR-ToF-MS analysis with NO^+ as reagent ion has allowed (i) the detection of aldehydes and ketones in separated peaks, (ii) to detect some molecules not found using H_3O^+, (iii) to confirm the results obtained using H3O+ as reagent ion for VOC analysis. VOCs results were thus confirmed by protein analysis according to qualitative and quantitative differences, which turn out to be able to differentiate samples within the capsicum genus. Moreover, the multivariate statistical approach revealed that some of these compounds can be successfully used for the species recognition in our artificial neural networks. The BPNN classifier utilized in the work had always 100% of success, both for H_3O^+ and NO^+ (data not shown) and this was probably due to the marked differences in the volatiles emissions of the three species. Further studies will aim to use the same method for a higher number of species, to challenge the analysis. Our investigation permitted the identification of 12 promising VOCs as more discriminant for each species and, among them, the masses *m/z* 81.068 and *m/z* 85.064 have been recognized as the most promising volatile markers of these species (Fig. 3). In addition to 12 VOCs, regarding the capsaicinoids, the DHC content was a more effective parameter to distinguish among domesticated and wild species of the genus *Capsicum* with respect to the capsaicin. The results from the random tree have been according with the protein analysis, which showed that the *C. chacoense* and *C. baccatum* have more similarities with respect to the C. annuum, although this was a preliminary analysis and additional analysis need to be done to support our hypothesis. According to our findings, both domesticated species, i.e. *C. baccatum* and *C. annuum* differ from the wild species *C. chacoense*, in particular, the *C. annuum* resulted in being the most dissimilar from the wild species. This was probably due to a more strict genetic selection that the *C. annuum* faced during years of domestication and supports the hypothesis that C. chacoense is one of the most ancient species of the genus and that the *C. baccatum* and *C. annuum* evolved separately from this common predecessor.

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Adv. Hort. Sci., 2021 35(3): 319-327 DOI: 10.36253/ahsc-10346

Review paper

AHS
Advances in Horticultural Science

Prospects for improvement of *Plectranthus edulis* (Vatke) Agnew: A high potential food security crop

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Key words: Genetic diversity, micropropagation, neglected crops, nutritional composition, tuber.

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Citation:
FEYISSA T., 2021 - *Prospects for improvement of Plectranthus edulis (Vatke) Agnew: A high potential food security crop.* - Adv. Hort. Sci., 35(3): 319-327.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Author Contributions:
All authors contributed equally to the work.

Competing Interests:
The authors declare no competing interests.

Received for publication 17 January 2021
Accepted for publication 16 July 2021

Abstract: *Plectranthus edulis* (Vatke) Agnew is a tuber crop originated and cultivated in Ethiopia for food and medicinal uses. It has higher quality nutrient composition than other tuber crops. The cultivation of *P. edulis* is under threat because of introduction of exotic species, land use change, habitat destruction, population pressure, selection of only few local varieties by farmers and climate change. Although *P. edulis* is a high potential food security crop, there is no any improved variety as there has been very little research on the crop. This lack of research attention including absence of improved varieties and lack of conservation strategy may lead to significant genetic erosion of this crop. Previous studies of *P. edulis* include documentation of indigenous knowledge, analysis of nutritional composition and the use of its starch in drug formulation, traditional cultivation practices, micropropagation, *in vitro* regeneration, and morphological and molecular genetic diversity. However, more extensive research in all aspects is required for its improvement, use and conservation. Therefore, it is imperative to review the research results of this crop for the research community. This article reviewed research findings of this crop and presents it in a comprehensive way so that the readers will get ample information and can refer original research findings for the details.

1. Introduction

Plectranthus edulis (Vatke) Agnew (synonym. *Coleus edulis*) is an ancient indigenous tuber crop that occurs both as cultivated and wild species in Ethiopia. Although it is known by different local names depending on the place where it is cultivated, *Oromo dinich* is the most popular local name. The cultivation of *P. edulis* is restricted to Ethiopia. It is mainly cultivated in the south and south western parts of the country at altitudes ranging between 1880 and 2200 m a.s.l. as food, and sometimes medicine (Demissie, 1988; Taye *et al*., 2007; Megersa, 2010). *P. edulis* is a dicotyledonous plant that grows up to a height of 1.5 m (Taye, 2008).

P. edulis is a high energy food crop and the tubers contain substantial amounts of micro- and macro-nutrients. When compared to Irish potato

(*Solanum tuberosum*), *P. edulis* has higher food energy and contains twice the amount of fat and calcium (EHNRI, 1997). It contains a similar amount of protein to that of Irish potato and almost twice that of sweet potato (*Ipomoea batatas*) after cooking (EHNRI, 1997).

The cultivation of *P. edulis* is under threat because of a decline in diversified local food production systems as a result of introduction of exotic species, land use change, habitat destruction, population pressure, selection of only a few local varieties by farmers, farming system intensification and climate change (Smolders, 2006; FAO, 2010). The decline in cultivation of the crop may result in erosion of the genetic base preventing the use of distinctive useful traits that are used for crop adaptation and improvement (Padulosi *et al*., 2002; IBC, 2005).

There is enormous untapped potential in Ethiopia to exploit the rich and diverse plant genetic resources of underutilized root and tuber crops including *P. edulis*. In spite of this potential, very little research has been done to improve the productivity of *P. edulis* (IBC, 2007). Currently, however, *P. edulis* is realized as a high potential food security crop that is attracting research attention.

The poor storability of tubers is found to be the major production constraint of *P. edulis*. It is propagated by vegetative methods so that it is susceptible to diseases that are transmitted from one generation to the next through infected planting material (Taye, 2008). This problem can be solved by integration of new technologies including biotechnological approaches. Documentation of information on indigenous knowledge, uses, and distribution of this crop is essential for its conservation, further utilization, and improvement (Mathenge, 1995). It is also important to initiate research on seed production of *P. edulis*. So far, there is no any improved variety of *P. edulis*. The objective of this article is to review some aspects of this crop, with particular regard to biotechnological approaches and present it in a comprehensive way so that the readers will get summarized information to design research or policy on conservation and improvement of the crop.

2. Production practices

Farmers select seed tubers from the previous harvest or they buy from the market. Most farmers plow the land more than three times before planting and mix farmyard manure with the soil (Mekbib and Weibull, 2012). Farmers do not use chemical fertilizers and other commercial inputs for production of *P. edulis*. The seed tubers are planted from March to April as there is brief rain during this time. Farmers commonly slice a single tuber into pieces and plant the pieces because they claim planting tuber pieces increases tuber yield (Taye *et al*., 2007; Mekbib and Weibull, 2012). Research findings also showed that planting tuber pieces results in higher yields than whole tubers (Taye, 2008).

In southern Ethiopia, almost 50% of the farmers plant the tubers with their sprouts whereas the others plant after removing the sprouts (Taye, 2008). Most *P. edulis* growing farmers remove the shoot apex with one or two pairs of leaves from the main stem and branches to increase stem number. Research results show that this practice significantly increases the number of stems and enhances faster stolen formation. Most farmers commonly build up the soil around the stem to enhance plant growth and increase tuber yield. The absence of positive correlation between frequency of removing the shoot apex and amount of the soil built up around the stem was reported, although both activities serve the same purpose (Taye, 2008).

Weeding is carried out at least three times at different growing stages of *P. edulis*. Following harvest of *P. edulis*, farmers plant other crops, and the yields of crops that follow *P. edulis* in the rotation are high. The reason for increase in yield could be due to the slowly released nutrients into the soil and improved soil structures as the result of the organic manure applied in previous cropping seasons (Mekbib and Weibull, 2012). Depending on the type of variety, *P. edulis* is harvested from six to eight months after planting. Although the cultivation is mainly performed by male farmers, female farmers also play important roles in selecting tubers for different end-use criteria related to the household food requirements (Mekbib and Weibull, 2012).

Farmers used to maintain several local varieties of *P. edulis* for different purposes. However, the number of local varieties preserved by farmers has been declining. So far, the total number of local varieties all over the country is not known. However, there are at least six local varieties of *P. edulis* that grow in specific areas as study results on three kebeles (the smallest administrative units in Ethiopia), namely Delebo-atewaro, Delebo-wogene and Kokete-marechare of Sodo Zuria district, South Ethiopia have

shown (Mekbib and Weibull, 2012). Growth habits, taste, tuber skin color, days to maturity, drought tolerance, storage period and marketability are the main characteristics of the plant used by farmers to identify local varieties (Mekbib and Weibull, 2012). Distribution and seed exchange of local varieties by farmers play key roles in conservation and utilization of local varieties (de Boef, 2008). Younger farmers mostly purchase the planting material from markets whereas older farmers (older than fifty years) use their own saved seed tubers from the previous harvest. Older farmers have much richer indigenous knowledge than younger farmers about the cultural value of the crop, indicating a limitation in the documentation of knowledge on the management. These knowledge gaps may lead to cultivation of only few local varieties (Negri, 2003; Mekbib and Weibull, 2012). This calls for more research on documentation of local knowledge and transferring this knowledge to the young farmers.

One of the major challenges of *P. edulis* production is seed tuber storage. The tuber is stored in the field where it is produced until used for the required purpose. This storage method results in significant tuber yield loss due to excessive heat especially during dry seasons. The land where the tubers are stored remains idle until all the tubers are removed. Therefore, as farmers do not want their land to be idle, most of them often obtain seed tubers from other sources during planting season (Mekbib and Weibull, 2012). Farmers cover *P. edulis* fields with mulching materials to protect the tubers from direct sunlight until the next planting season. After the preparation of the land for planting, tubers are transferred to the pits for a day to facilitate the germination and performance of seedlings. Most seed tubers are obtained from farmers who have enough land to produce *P. edulis* as they produce more than a household requirement and save the surplus for sale (Negri, 2003). Although different cultural practices have been designed to overcome the production constraints of *P. edulis*, very little research has been done to tackle the problems. Another important constraint is shortage of land for planting (Mekbib and Weibull, 2012). This can be solved by amending land usage policy.

Some efforts have been made to evaluate performance of *P. edulis* with regard to yield and yield related characters and reports show different performance of the crop at different environments (Taye *et al*., 2013). Tuber fresh weights of 4556-4933 g m^{-2}, or 45-49 Mg ha^{-1} were obtained at experimental sites, which is higher than yields produced by farmers (740-1480 g m^{-2}). This higher experimental yields compared to farmers' yields is because farmers harvest the tubers early Late emergence, slow development of the canopy, full coverage of the ground in a short period of time and decline in soil cover during senescence caused poor radiation interception. Radiation interception of a crop can be enhanced by increasing ground cover by the canopy. This could be achieved by planting large seed tuber pieces (Wiersema and Cabello, 1986; Lommen and Struik, 1994), by planting more tuber pieces per area (Spitters, 1990) or by using traditional practices that enhance the canopy development (Taye *et al*., 2012).

3. Nutritional content

P. edulis contains substantial amounts of nutrients. Moisture content of the tuber dry matter ranges from 14.1% to 17.5%, whereas the protein content ranges from 0.70% to 1.76% (Hellemans *et al*., 2017). These values are higher than cassava tuber, which contains 0.3% protein and 0.1% to 0.3% starch (Alvani, 2011; Waterschoot *et al*., 2015). Another study reported 11.2% moisture, 0.14% ash, 0.21% lipid, 0.43% protein, and 99.22% starch content on a dry weight basis (Assefa, 2015) (Table 1).

Table 1 - Proximate composition of *Plectranthus edulis* tubers on dry weight basis in g per 100 g

Sample type	Moisture content (%)	Crude protein	Total ash	Crude fiber	Crude fat	Carboydrate	Energy (Calories)	References
Raw	81.9	1.5	1.1	0.70	0.20	15.3	69	EHNRI [(z)], 1997
Boiled	73.8	1.0	1.3	1.00	0.20	23.7	101	EHNRI, 1997
Starch	11.2	-	0.14	-	0.21	99.2	-	Assefa, 2015
Starch	15.5	1.09		3.0	0.24	-	-	Alvani *et al*., 2011

[(z)] Ethiopian Health and Nutrition Research Institute

Samples collected from different regions of Ethiopia showed variation in fiber content based on their geographic location. Starch extracted from tuber samples collected from Wolaita zone (Chenqoua, Inuka and Lofua areas) showed significantly lower fiber content than tuber samples collected from Arjo white, Chencha and Jarmet. Moreover, the samples collected from Arjo white, Chencha and Jarmet contain higher fiber content than cassava or potato. Whether this variation is due to the environment or genetic variation needs further investigation. *P. edulis* contains amylase ranging from 14.2% to 23.9% (Waterschoot *et al*., 2015).

Tuber samples collected from Abay Chomen district of Oromia Region, Ethiopia, showed variation in mineral content may be due to genotype or environmental variation. *P. edulis* is found to be rich in potassium followed by magnesium, calcium, zinc, manganese, and copper. Mineral content of *P. edulis* is presented in Table 2. The trace metals (chromium and nickel) and the heavy metals (lead and cadmium) were not detected, indicating *P. edulis* is safe with regard to the toxic heavy metal health concerns (Lema, 2016). The phosphorus content of most *P. edulis* samples is twice as much as in Irish potato. High phosphorus content is linked to resistant starch, paste viscosity and gel strength (Lu *et al*., 2012). Higher phosphorus content is responsible for the granular structure, and especially for the crystalline region to be less rigid and therefore enabling absorption of more water (Lin *et al*., 2013). High phosphorus content was also found to impart high viscosity to the starch and increase gel strength. This indicates *P. edulis* starch can be used in food processing that requires high gel strength and can provide resistant starch in functional food preparation (Hellemans *et al*., 2017). However, further research should be conducted to confirm this finding. *P. edulis* is found to be richer in most of the mineral elements than Irish potato.

Starch physicochemical properties

Scanning Electron Microscopy (SEM) of the starch granules showed elliptical and some oval shapes. The starch showed a normal monomodal granule size distribution. X-Ray Diffraction pattern of the starch showed typical B-type with a distinctive peak (Assefa, 2015). On the contrary, other authors reported distinct bimodal distribution pattern of starch granular characteristics, which indicates the presence of small (B-type) and large (A-type) granules (Hellemans *et al*., 2017). The shape of B-type granules is spherical whereas that of A-type is elliptical when visualized by the cryo-SEM. For starch extracted from *P. edulis* tuber, such bimodal distribution is not common. Starch extracted from Irish potato and cassava shows normal distribution pattern (Waterschoot *et al*., 2015). Therefore, *P. edulis* can be used in food industry and as an alternative for potato starch, which is less suitable to be used in pasteurized foods.

Typical water absorption (WA) pattern, swelling power and relative humidity of *P. edulis* is reported to be higher than Irish potato starch but its solubility at all studied temperatures is lower than Irish potato (Assefa, 2015). The physicochemical analysis of the starch showed 1.08 g/g WA of *P. edulis* (Hellemans *et al*., 2017). This means that the starch granules absorb on average of 1.08 times their own weight after it is completely hydrated. Only a small difference was observed in WA among *P. edulis* starch samples of different accessions. The high water binding ability of the starch could reduce the stickiness of dough, increase moistness, improve handling and soften the texture of baked products, which makes *P. edulis* starches more suitable for use in baked goods (Taggart, 2004).

Differential Scanning Calorimeter (DSC) thermograms of *P. edulis* starch showed higher onset temperature (To) of 69.2°C, peak temperature (Tp) of 74.3°C and ending temperature (Te) of 83.3°C than the starch of Irish potato (Assefa, 2015). Similarly, the pasting properties showed *P. edulis* starch has

Table 2 - Mineral content of *Plectranthus edulis* in mg per 100 g

Sample	K	Ca	Fe	Mg	Zn	P	References
Raw	-	29.0	9.30	-	-	90.0	EHNRI, 1997
Boiled	-	19.0	1.10	-	-	62.0	EHNRI, 1997
Starch [w]	513	397	20.8	175	4.5	1803	Alvani *et al*., 2011
Starch	3.54	0.62	-	0.49	0.24	-	Lu *et al*., 2012

[w] ppm/dm- parts per million/dry matter.

higher pasting temperature than Irish potato (Hellemans *et al.*, 2017). A rapid increase in viscosity occurred at Tp range of 70.7 to 74.0°C compared to Irish potato starch (52.2°C).

The disintegrating abilities of starch of *P. edulis* and Irish potato were compared in paracetamol tablet formulations prepared by wet granulation method (Assefa, 2015). The granules were prepared with the starches at different disintegrant concentrations and characterized for particle size distribution and flow properties. The prepared tablets showed excellent flow property as manifested by the weight uniformity. After studying the crushing strength, friability, disintegration time, and dissolution rate of the tablets using standard methods, the results showed that *P. edulis* starch favorably competed with potato starch as a disintegrant in the paracetamol tablet formulations.

In other recent study, carboxymethylated *P. edulis* starch was evaluated as a suspending agent in metronidazole benzoate suspensions by comparing with sodium carboxymethyl cellulose (NaCMC) (Brhane, 2020). The author reported viscosities of the formulations prepared with carboxymethylated *P. edulis* starch were significantly lower than that of NaCMC. The flowability of the suspensions was in the order of carboxymethylated *P. edulis* starch (CMPS) greater than NaCMC. At 1% concentration, carboxymethylated *P. edulis* starch resulted in significantly higher sedimentation volume than NaCMC. Potassium dihydrogen phosphate (KH_2PO4), which is employed as a flocculating agent, significantly increased the sedimentation volume of the suspensions prepared with carboxymethylated *P. edulis* starch and NaCMC. The redispersibilities of CMPS were found to be better than NaCMC. The author pointed out that all suspensions resulted in a release of greater than 85% of drug within 1 h. All suspension formulations were found to be stable (Brhane, 2020). The author concluded carboxymethylated *P. edulis* starch can be used as alternative suspending agent.

4. Prospects of biotechnology for improvement of *P. edulis*

In addition to the shortage of seed tubers and the poor storability of the tubers, systemic diseases, viruses, viroids and mycoplasma as well as several pathogenic bacteria are the most devastating root and tuber crops including *P. edulis*, in terms of yield loss (Bryan, 1983). These problems might be solved by using the benefits of modern plant biotechnology such as plant tissue culture that can supply a large number of disease-free planting materials to growers who continually acquire it.

Micropropagation and in vitro *shoot regeneration*

Increased use of rapid multiplication techniques enables production of large amounts of pathogen-free planting material. Most rapid multiplication techniques in root and tuber crops involve the use of aerial portions of the plant so that contact with soil and tuber is broken and most non-systemic pathogens can be eliminated (Bryan, 1983). Meristem culture is the most popular tissue culture technique for production of disease free planting materials. It can be used alone or in combination with thermotherapy to eliminate a number of viruses and bacteria (Kartha and Gamborg, 1975). Meristem culture technique eliminates systemic pathogens found in the mother stock plant.

The first micropropagation protocol of *P. edulis* was developed from meristem culture (Tsegaw and Feyissa, 2014) followed by *in vitro* shoot regeneration from leaf derived callus (Aschale and Feyissa, 2019). Shoots were initiated from meristem explants using Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium. The MS medium was supplemented with 1.0 mg/l gibberellin (GA_3) and 0.1 mg/l α-naphthalene acetic acid (NAA) in combination with different concentrations of benzylaminopurine (BAP) (Tsegaw and Feyissa, 2014). According to the authors, MS medium containing the aforementioned hormones was proved to be the best in terms of shoot initiation from meristem for mother plants collected from two locations, Holeta and Wolaita. The shoots were transferred to multiplication medium and the highest mean shoot number per explant (7.2) was obtained on medium containing 1.0 mg/l kinetin in combination with 0.1 mg/l NAA for shoots derived from mother plants collected from Holeta while, 6.2 shoots per explant was obtained on medium containing 3.0 mg/l kinetin in combination with 0.05 mg/l NAA for shoots derived from mother plants collected from Wolaita. The effect of solid and liquid media on *in vitro* axillary shoot proliferation was investigated by culturing shoots on MS medium containing different concentrations of BAP in combination with thidiazuron (TDZ) (Yimam, 2013). The highest mean shoot number per explant, 5.85 and 6.07, were obtained on solid and liquid media,

respectively, containing 1.5 mg/l BAP in combination with 0.5 mg/l TDZ.

Developing *in vitro* regeneration protocol through callus phase or somatic embryogenesis is a prerequisite to genetically improve a crop through biotechnological approaches including genetic engineering, genome editing and creating somaclonal variants. Recently, *in vitro* regeneration protocol of *P. edulis* have been developed by using leaf explants (Aschale and Feyissa, 2019). The highest percentage of callus induction (100%) was obtained on MS medium supplemented with 1.5 mg/l NAA in combination with 1.0 mg/l BAP and 2.0 mg/l NAA in combination with 0.5 mg/l BAP. The highest shoot regeneration percentage (46.6%) from callus was obtained on MS medium containing 0.1 mg/l BAP in combination with 0.5 mg/l TDZ. The highest mean shoot number per callus (1.66) was obtained on medium containing 1.5 mg/l BAP in combination with 1.0 mg/l TDZ. The highest mean shoot length (0.63 cm) was obtained on medium supplemented with 0.5 mg/l BAP in combination with 0.1 mg/l TDZ. The highest mean shoot number per explant (58.68) was obtained on medium supplemented with 0.5 mg/l BAP in combination with 0.4 mg/l GA3.

Rooting and acclimatization

Shoots cultured on half salt strength MS medium rooted better than those cultured on full salt strength (Tsegaw and Feyissa, 2014). Shoots derived from explants collected from Holeta resulted in the highest percentage of rooting (100%) and 3.12 mean root number per shoot whereas shoots whose explants were collected from Wolaita resulted in 76.7% rooting and 2.2 mean root number per explant. Microshoots were also directly planted in a greenhouse for rooting and acclimatization by bypassing the *in vitro* rooting stage and the overall best result in rooting was obtained in *ex vitro* rooting condition. It was promising in cost reduction as it bypasses the *in vitro* rooting stage. Reducing the cost of *in vitro* production is a key for increasing the application of the method. Rooting of microshoots *in vitro* is expensive and can even double the price of the plantlets (Zimmerman, 1988; De Klerk, 2002). In another study, shoots were cultured on half strength MS medium containing different concentrations of indole acetic acid (IAA) and indole butryic acid (IBA) for rooting (Yimam, 2013). The highest mean root number per shoot (10.55) was obtained on solid medium and 13.11 mean root number per shoot was obtained in liquid medium containing 0.1 mg/l IAA. The highest mean root number per shoot of 10.15 on MS medium supplemented with 2.0 mg/l IBA was also reported (Aschale and Feyissa, 2019).

All *in vitro* regenerated plants of Holeta origin and 96% of Wolaita origin survived after one month of acclimatization in a greenhouse. Similar study reported 100% and 82.85% survival of plantlets derived from solid and liquid media, respectively, after acclimatization in greenhouse (Yimam, 2013). All *in vitro* rooted and 78 % *ex vitro* rooted microshoots survived in the greenhouse (Aschale and Feyissa, 2019).
The development of these protocols for micropropagation and *in vitro* regeneration of shoots from callus is the prerequisite for further advanced biotechnology research such as genetic transformation and genome editing of this crop.

5. Genetic diversity of *P. edulis*

For improvement of any crop, the existence of adequate genetic diversity is imperative. This could be achieved if and only if there is appropriate conservation strategy. Selection and cultivation of only few local varieties of *P. edulis* and its current replacement by other tuber crops has been causing serious genetic erosion of the local gene pool of this crop.

Genetic diversity analyses using morphological traits

There are several studies on morphological genetic diversity of *P. edulis*. Genetic diversity of 36 accessions of *P. edulis* using 16 morphological traits was assessed and highly significant variation among the accessions for all the analyzed traits except length of tuber was reported (Garedew *et al*., 2013). In another study, 20 *P. edulis* accessions that were assessed using 29 morphological traits showed 75.7% variance among the accessions (Mekbib, 2007). This study showed that there are accessions collected from different regions of the country but showed genetic similarity, and some of those collected from the same geographic area showed different genetic background. This indicates geographic diversity does not necessarily represent genetic diversity. Similarly, genetic diversity analysis of 20 accessions using 13 traits revealed significant variation of all traits except flower length and leaf width (Soresa, 2017). Recent extensive genetic diversity study of 174 accessions at three environments based on 12 qualitative and 16 quantitative traits also showed a highly significant

variation among the populations and non-significant environment-population interaction for most of the quantitative traits (Gadissa *et al*., 2020). The first six principal axes of principal components analysis accounted for 77% of the total variation. The populations were grouped into four clusters but there was no strong groupings based on geographical locations from where the genotypes were collected. This indicates the historical or contemporary gene flow, particularly tuber exchange among the different geographical regions. Although there are reports of relatively high genetic diversity, still there is a need for more collections and evaluation over multiple locations and seasons for well-refined genetic diversity estimation. However, as the analyzed traits are affected by the environment, the morphological genetic diversity results should be complemented by molecular genetic diversity studies.

Genetic diversity analyses at molecular level

Assessment of molecular genetic diversity of *P. edulis* was conducted by using inter simple sequence repeat (ISSR) markers for the first time in 2015 (Shiferaw, 2015). The Shannon information index ranged from 0.25 to 0.39 with overall index of 0.48. Nei's gene diversity ranged from 0.18 to 0.30 with overall mean diversity of 0.33, and 74.23% within population variation. Analysis of Molecular Variance (AMOVA) showed within population diversity range of 41.30% to 65.22%. Another study using the same marker exhibited overall percent polymorphism of 95%, 0.62 Shannon information index and 0.40 Nei's gene diversity (Gebrehiwet *et al*., 2019). More extensive study of genetic diversity assessment was conducted by using expressed sequence tag simple sequence repeats (EST-SSRs) derived from *P. barbatus* (Gadissa *et al*., 2018). The author identified 128 alleles in 12 populations that consisted of 287 individual plants. The results showed gene diversity index range of 0.31 to 0.39 with overall mean of 0.35. Among the 12 populations, Wenbera, Awi and Wolaita populations showed the highest genetic diversity, and hence these populations can be considered for *in situ* conservation and identification of genotypes with traits of interest that can be used in breeding programs. AMOVA showed low population differentiation with only 3% of the total variation accounting for variation among populations. STRUCTURE and cluster analyses did not group the populations into distinct clusters, which may be attributed to historical and contemporary gene flow and also the reproductive biology of the crop.

6. Conclusions

P. edulis is a high potential food security crop containing substantial amounts of nutrients. However, there is no any improved variety of the crop, and currently its cultivation is declining. Shortage of tuber seed is the major problem of this crop. Recently, the farmers are turning to produce other cash crops that generate more income. With advances in plant research, it is important to integrate both conventional and modern technologies to improve this crop. Unless urgent attention is given to such neglected and underutilized crops, these crops are extremely subjected to genetic erosion as the farmers shy away from producing them. Using biotechnological research tools such as plant tissue culture, genetic engineering, genome editing and plant molecular breeding might be a future hope for improvement of this crop, although the international funding opportunities for such orphan crops are highly limited. *In vitro* propagation and *in vitro* regeneration protocols from different explants have already been developed as reviewed in this article and these protocols can be used for improvement of *P. edulis*. In addition, information from the genetic diversity results reported so far could be used as a baseline for more research in the area and improve this crop using genomic tools including marker assisted breeding.

Acknowledgements

The author acknowledges Addis Ababa University for providing the necessary resources during writing of this review. The author is also grateful to graduate students who have been working with him and other researchers who generated interesting information on *Plectranthus edulis* that served as resources to write this review.

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Adv. Hort. Sci., 2021 35(3): 329-332 DOI: 10.36253/ahsc-10854

Short note

Artificial medium for *in vitro* pollen germination of some ornamental *Linum* species

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Key words: Artificial medium, *in vitro* germination, Linum, pollen, polyethylene glycol, sucrose, wild species.

Abstract: For the first time components of the nutrient medium were identified for the successful germination of pollen in such ornamental species of the *Linum* genus as *L. grandiflorum*, *L. hirsutum*, *L. pubescens* and *L. thracicum*. It was shown that the media with PEG-2000 in concentrations of 20-30% in combination with boric acid and calcium chloride in the concentrations of 200 mg/l ensure germination of *Linum* pollen up to 40-50%. The addition of sucrose and PEG with higher molecular weight adversely affects the germination of pollen. This will allow anyone to evaluate the quality of flax pollen quickly and efficiently and more successfully implement many genetic and breeding programs.

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Citation:
LYAKH V.A., SOROKA A.I., 2021 - *Artificial medium for in vitro pollen germination of some ornamental linum species.* - Adv. Hort. Sci., 35(3): 329-332.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 5 May 2021
Accepted for publication 21 August 2021

1. Introduction

The *Linum* genus has between 100 and 230 species with the main representative of *Linum usitatissimum* L., which is one of the oldest cultivated plants, whose products have long been used for a variety of human needs. Not less diverse is the use of wild flaxes (Jhala *et al*., 2008; Lyakh and Soroka, 2008). Moreover, some annual and perennial wild relatives of the *Linum* genus, such as *L. grandiflorum*, *L. pubescens*, *L. hirsutum*, possessing fairly large flowers of various colors, are highly ornamental plants and are successfully applied in landscaping, flower bed arrangement, and gardening (Lyakh, 2013).

A number of wild Linum species is a producer of valuable substances for the pharmaceutical industry (Mohammed *et al*., 2009). Ushijima *et al*. (2012) reported that many wild flax species exhibit distinct floral polymorphism, which allows them to be used for elucidation the mechanisms of such a phenomenon as heterostyly. Wild flax relatives are also actively involved in phylogenetic analysis of the *Linum* genus (Muravenko *et al*., 2009; Sveinsson *et al*., 2014).

A broad use of wild flax species and active breeding work with them provides for knowledge of the quality of pollen, produced by the plant. The ability of pollen to grow and germinate on an artificial medium allows estimating its quality fast and effectively (Jayaprakash, 2018).

In flax, separate attempts were made to germinate pollen *in vitro* by Pandey and Kumar (2013). However, for the pollen of both cultivated flax and its wild relatives, the medium, which ensures the emergence of properly-shaped pollen tubes during pollen germination, has not yet been developed. In this respect the purpose of this work was to develop a nutrient medium suitable for the germination of pollen from a number of ornamental flax species.

2. Materials and Methods

Wild species *L. grandiflorum* Desf., *L. hirsutum* L., *L. pubescens* Banks and Solander and *L. thracicum* Degen were used in our studies as pollen sources. Experiments were carried out during 2017-2018.

The medium containing boric acid and calcium chloride in the concentrations of 200 mg/l was used as a basic one. A medium, consisting of boric acid, calcium chloride and sucrose as osmotic agent is commonly used for pollen germination of different species. We, however, excluded sucrose as according to our preliminary experiments with *Linum* species it inhibited completely pollen germination. Polyethylene glycol (PEG) of various molecular weights was supplied to the basic media (boric acid and calcium chloride) as osmotic agent. In some cases we also used sucrose as an addition to PEG. The following additions to the basic medium have been made: (a) PEG 2000, 20%; (b) PEG 2000, 30%; (c) PEG 2000, 30% + sucrose, 5%; (d) PEG 2000, 30% + sucrose, 15%; (f) PEG 6000, 30% + sucrose, 5%; (g) PEG 20000, 5% + sucrose, 15%.

Pollen was collected from 20-40 flowers and germinated for 3-4 hours in a drop of an artificial medium placed on a slide at the temperature of 25±1 °C in the dark. The pollen was then viewed under a light Leica microscope (Germany) with a 20X objective. Pollen grains were counted as germinated if the pollen tube length was more than a pollen grain diameter. In each 3-5 replication of each treatment several fields of view were analyzed to count from 300 to 400 pollen grains. Pollen grains near the margin of the medium were not recorded. After that a mean value of pollen germination percentage and a standard error of the mean were calculated (Lyakh and Soroka, 2008).

The results of the experiments were analyzed statistically applying a t-test, according to Wasserman (2005).

3. Results and Discussion

Figure 1A shows that a medium containing PEG-2000 as an osmotic in the concentration of 20% ensured a sufficiently good germination of the pollen for the species under study. The percentage of germinated pollen grains ranged from 22.9±2.44 in *L. thracicum* to 51.0±2.84 in *L. grandiflorum*. The elevation in concentration of PEG-2000 from 20% to 30% did not reduce this indicator in all species, except *L. hirsutum*, where an increment in the pollen germination was observed. Addition sucrose to the

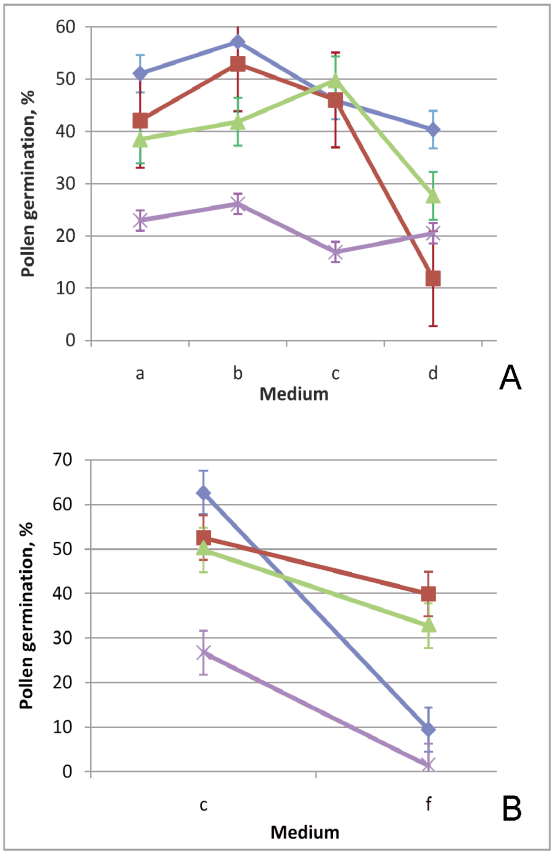

Fig. 1 - Influence of PEG-2000 concentration and sucrose addition (A) and PEG molecular weight (B) on pollen germination of some ornamental *Linum* species: --♦-- *L. grandiflorum*, --■-- *L. hirsutum*, --▲-- *L. pubescens*, --X-- *L. thracicum*.

nutrient medium with PEG-2000 adversely affected pollen germination as a whole. Elevating the sucrose concentration up to 15% in a medium with PEG 30% reduced the number of germinated grains in all the species. Figure 1B data grant an idea on the influence of PEG with different molecular weight on pollen germination. As revealed, the percentage of pollen germination was significantly larger in the case of an osmotic with a lower molecular weight. It is characteristic that the number of germinated pollen grains on a medium supplied with PEG-6000, compared to PEG-2000, for *L. grandiflorum* and *L. thracicum* decreased by a factor of 6.6 and 20.5, whereas for *L. pubescens* and *L. hirsutum* - the reduction amounted to 34.0 and 24.1% only.

Figure 2 demonstrates pollen germination pattern on a media with PEG of different molecular weight and sucrose, showing the proportion of pollen grains with normal and burst pollen tubes. It can be seen that the percentage of pollen grains with burst tubes both in *L. grandiflorum* and *L. hirsutum* on a medium containing a high molecular weight polyethylene glycol (PEG-20000) at the concentration of 5%, against the background of 15% sucrose, was quite large. It is characteristic that for some species it even exceeded the number of pollen grains with normal tubes. When the medium included PEG-20000 at the concentration of 30%, pollen of all the studied species failed to germinate.

As can be seen, in our experiment sucrose negatively affected the germination of pollen when it was added to the media with PEG. With sucrose concentration increasing, the number of germinated pollen grains decreased notably. At the same time there are successful examples in the literature of the joint use of sucrose and PEG. Thus, for sunflower a nutrient medium was developed suitable for pollen germination which simultaneously included 15% of sucrose and 30% of PEG (Keshava Murthy *et al.*, 1994). Such medium was successfully used to evaluate pollen response of various sunflower genotypes to the action of low temperature while selecting pollen for cold resistance (Lyakh and Totsky, 2014).

Analyzing the pollen germination of ornamental flax species on the media with PEG of different molecular weights, it is clearly noticeable that with an increase in the PEG molecular weight the degree of reduction of the studied indicator was different for different species. It can be assumed that such a difference in pollen response is due to the different osmotic potential of the pollen grains considering the natural habitat and presence a number of xeromorphic traits in *L. hirsutum* and *L. pubescens* as apposed to *L. grandiflorum* and *L. thracicum* (Tutin *et al*., 1968).

Pandey and Kumar (2013) have investigated *in*

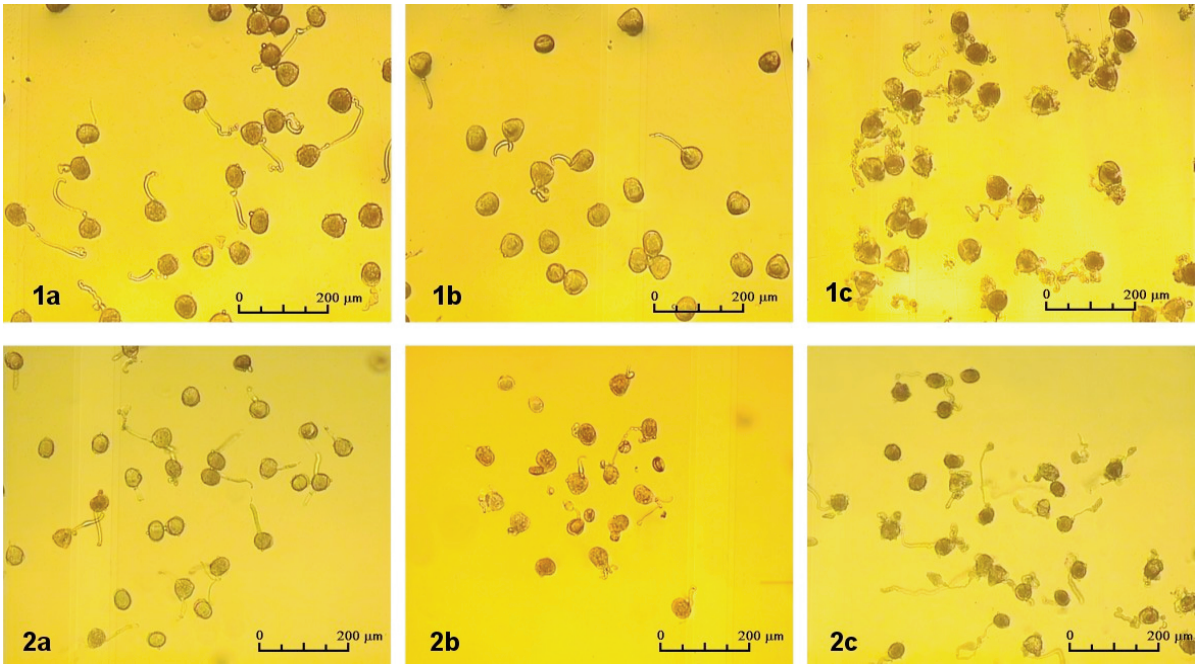

Fig. 2 - Pollen germination of *L. grandiflorum* (1) and *L. hirsutum* (2) on the media with PEG of different molecular weight and with sucrose: (a) PEG-2000, 30% + sucrose, 5% (b) PEG-6000, 30% + sucrose, 5%; (c) PEG-20000, 5% + sucrose, 15%.

vitro pollen germination in *L. usitatissimum* on a medium containing as an osmotic only sucrose. However, despite the high ability of the pollen of this species to germinate under the given conditions, pollen grains emitted mostly pollen tubes with malformed morphology. In our experiment pollen of ornamental flax species germinated well and developed normal pollen tubes on the media containing, in addition to the basic components, PEG-2000 as an osmotic agent.

4. Conclusions

Wild species of the genus *Linum* are widely represented on the ornamental plant market. Moreover, their relatives can be used as a source material for fiber and oil flax breeding. For genetic and breeding programs pollen quality assessment is an important, and often necessary, procedure for their successful implementation. *In vitro* germination of pollen on artificial nutrient media is the simplest, but at the same time reliable way to determine the pollen viability. Our studies have shown that pollen of some wild species of the genus *Linum* germinates well on the media containing, in addition to boric acid and calcium chloride, an osmotic agent in the form of polyethylene glycol-2000. Replacing this osmotic with polyethylene glycol of a higher molecular weight or adding sucrose significantly impairs pollen germination rates. The patterns revealed allow to propose the composition of an artificial nutrient medium for germinating flax pollen, which will ensure its better germination than is known from the available scientific literature.

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