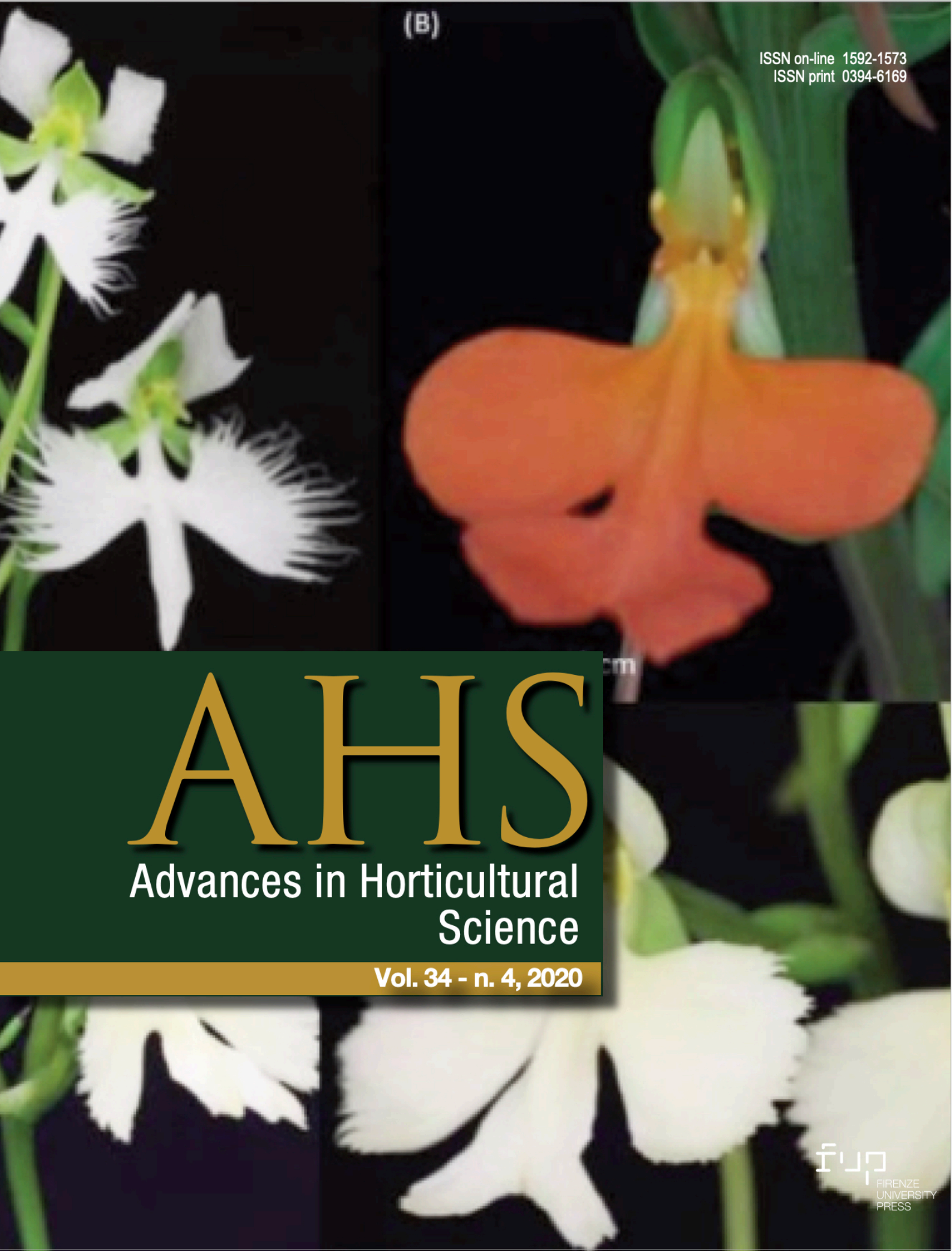

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Management of root knot nematodes (*Meloidogyne sp.*) and enhancing growth yield of greenhouse produced tomatoes by using fresh plant derived soil amendments

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OPEN ACCESS

Key words: *Lippia kituensis*, *Meloidogyne* sp., *Ocimum gratissimum*, organic amendments, tomato yield.

Abstract: Production of greenhouse tomato is hampered by myriad of challenges emanating from growth medium in the sub-Saharan Africa (SSA), which has led to instability in the production trend. A greenhouse experiment was conducted to study the effect of soil amendment with fresh plant biomass from *Lippia kituensis* Vatke and *Ocimum gratissimum* L. aimed at managing root knot nematodes (RKN) and enhancing tomatoes yield. The amendments were applied at 0 (soil negative control), *Lippia* and O*cimum*, each at 200 g, 400 g % and 800 g in 10 kg potted soil mixes, singly and in all possible combinations. Azadirachtin (0.3 w/w) was also used as a positive control. The mixtures were treated inoculums carrying 1000 second instar *Meloidogyne* sp. juveniles. An unbalanced factorial in a Randomized Complete Block Design with 3 replications was used. The parameters measured were nematode populations, root gall numbers, galling index, tomato growth, development and yield. Results indicated that interactive effect of soil amendment at 800 g of both *Lippia* and *Ocimum*, significantly ($p<0.05$) reduced the RKN population by 82.1% compared to the non-amended soil. At same rates, galls were reduced by 95.5% while galling index by 83.3%, compared to non-amended treatment. In plant development same amendment rates demonstrated higher vegetative growth. For fruit number and marketable yield, 76.7% and 82.2% more fruits per plant were recorded from 800 g LK+ OG at 800 g and Azadirachtin respectively, compared to non-amended soil. Based on the results, *Lippia* and *Ocimum* may be potential sources for nematicidal plant products for greenhouse tomato production.

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1. Introduction

Tomato is among the leading greenhouse vegetable crops grown in Kenya in both soil and soilless media (HCD, 2012). Tomato constitutes 7% of the total horticultural produce in Kenya and 14% of all the entire vegetable produce (Ochilo *et al.,* 2019). In terms of production in the year

2018, Kenya was rated 7th amongst the leading countries in sub-Saharan Africa with 599,458 tonnes per year, though the trend has not been stable (FAO-STAT, 2018). To stabilize the trend of production, greenhouse technology is seen as one of the solutions in tomato production, due to high levels of efficiency and the potential to support sustainable socio-economic development. However, greenhouse tomato production has not been vibrant in Kenya as it should be in the tropical humid region (Sanzua *et al.,* 2018). This is because under soil-based production, there is nutrient depletion through plant uptake and leaching beyond the root-zone of vegetable crops (Kirimi *et al.,* 2011), resulting in reduced yield in the following season. Besides, the continuous growing of tomato in the greenhouse soil leads to accumulation of soil borne pests and pathogens especially bacterial wilt *Ralstonia solanacearum*, Fusarium wilt, and Nematodes (*Meloidogyne* sp.), which has forced most farmers to abandon greenhouse tomato production (HCD, 2017).

Studies have shown that soil based media with appropriate amendments combined with other additives may effectively manage soil-born pests and diseases (McSorley, 2011). Among the pests Root-knot nematodes (RKN) *Meloidogyne spp* are the most damaging nematodes in tomatoes grown in the tropics (Walker, 2007). Infection of roots with *Meloidogyne* initiates a series of events that changes the entire physiology of the host plant. Root galls result when nematodes penetrate the cells of the cortex and pericycle the endodermis and reach the stale. About 5-7 cells surrounding the nematode's head enlarged to become a specialized giant cells, much larger than others. The thick nuclei enlarge, become polyploidy and undergo series of synchronized division, (Mai and Mullin, 1996). Root symptoms may appear as root knots (root galls), root lesions, excessive root branching (Ogallo *et al*., 1997) as root tips are injured and roots rot when nematode is accompanied by plant pathogenic or saprophytic bacteria of fungi (Cerkaukas, 2004). These nematodes are prevalent in the greenhouse condition and invade almost all vegetable crops resulting in substantial yield losses (Stirling and Stirling, 2003). Bekal and Becker (2000) observed that at peak, 100 cm^3 of soil contained 1000 nematodes. This declines with weather variation to approximately 50 nematodes per 100 cm^3 of soil. According to Stirling and Kopittke (2000), the economic threshold of RKN on most crops range between 2 and 10.

On yield various authors have reported on reduction in tomato due to RKN *Meloidogyne spp* ranging from 28% to 68% (Safiuddin Shahab *et al.,* 2012). Under soil based greenhouse tomato production, nematode infestations are a serious constraint leading to a yield reduction (Pakeerathan *et al*., 2009). Suppressed plant growth and yield has been observed in nematode infested fields (Vovlas *et al.*, 2005). Many crops grown as vegetables are susceptible to *M. incognita* and *M. javanica* particularly tomato, aubergine, okra, cucumber, melon, carrot, gourds, lettuce and peppers (Varela *et al*., 2003).

It has been suggested by McSorley (2011) that reduction of nematodes in fields treated with plant biomass wastes results in improved soil structure, fertility and improvement of plant resistance from nematode toxins. Similarly, it may increase fungal and bacterial parasites population in the soil or other nematode antagonistic agents. Apart from nematode control, these plant based biomass also provide essential nutrients (such as N and P), which help to rebuild soil organic matter contents, and aid in the re-establishment of beneficial microbial populations (Suresh *et al.,* 2004; Allen *et al.*, 2007; Dauda *et al*., 2008). Additionally, higher organic matter content increases soil water holding capacity and supports thriving communities of decomposers and predators in the soil system. The nematicidal properties of plants may be a contribution from extracted biomolecules resident in the plant bodies. The major classes of compounds with proven nematicidal activity include alkaloids, fatty acids, glucosinolates, isothiocyanates, phenols, diterpenes and a variety of essential oils (Chitwood, 2002). Neem cake, known to be rich in Azaderachtin is also associated with strong nematicidal activity (Abbasi *et al.,* 2005). Laboratory extracted essential oils have also been reported to affect development of nematode eggs and second juvenile stage (J2) under *in vitro* conditions (Onifade, 2007). Other extracts with strong pesticidal properties include rotenone, nicotine and pyrethrins (Berger, 1994).

In Verbenaceae, *Lippia* is among the genus of aromatic plants due to their essential oils (Kosgei *et al*., 2014). They are shrubs or woody herbs, leaves opposite or verticilate, glandular. Flower is pedunculate, crowded spikes; corolla obscurely 2- lipped, fruits are 2 dry mericarps each with very small seed. Various species in Kenya include; *L. dauensis* (Chiov.) Chiov, *L. grandifolia* A. Rich, *L. javanica* (Birm. F.) Spreng, *L. kituensis* Vatke, *L. somalensis* Vatke (Beentje, 1994).

The species *L. kituensis* Vatke has opposite leaves, ovate or elliptic; flowers white with yellow throat. In a previous study by (Kosgei *et al*., 2014), phytochemical analysis from methanol extracts of *Lippia kituensis* Vatke were done and reported to possess monoterpenes, Sesquiterpenes, diterpenes and other essential oil which were found to be effective against larvae *of Rhipicephalus appendiculatus*. Some of the these essential oils included Alpha-pinene (-)-, Camphene, Sabinene, beta-myrcene, l-Phellandrene, Dl-limonene, Gamma-terpinene, Trans-sabinene hydrate, Alpha-terpinolene, Neo-allo-ocimene, Camphor (1S 4S)-(-)-, Camphore, borneol (=endo-borneol), 4-methyl-1-(1-methylethyl)- 3-Cyclohexen-1-ol, 4-terpineol. In the same extract, Sesquiterpenes yielded were; Beta-bourbonene, isopropyl-5-methyl-9-methylene- Bicyclo[4.4.0]dec-1-ene, Germacrene D, Gamma-Cadinene, 2-isopropyl-5-methyl-9-methylene- Bicyclo[4.4.0]dec-1- ene. Duschatzky *et al*. (2004) reported that nematicidal activity of the essential oils isolated from *Lippia juneliana* and *L. turbinata*, which were evaluated using *in vitro* experiments. In another study, the oils of *L. juneliana* and *L. turbinata* showed the highest nematicidal activity among the tested oils, killing more than 80% of the juveniles of the *Meloidogyne* sp. Analysis of the oils revealed that *L. juneliana* contain; piperitenone oxide (36.5%), limonene (23.1%), camphor (8%) and spathulenol (6.5%) and *L. turbinata* has limonene (43.3-60.6%) and piperitenone oxide (39.3- 17.8%).

The genus *Ocimum* L. (Lamiaceae) comprises 30-160 annual and perennial herbs and shrubs, collectively called basil. Species of this genus are popular sources of essential oils and aromatic compounds, of condiments, and ornamental plants (Nagai *et al*., 2011). The most cultivated species worldwide are *O. africanum* Lour. *O. americanum* L., *O. basilicum* L., *O. gratissimum* L., O. *minimum* L. and O. *tenuiflorum* L., mainly due to their economic and medical importance (Carović-Stank *et al*., 2010). Essential oils in *Ocimum* incude Linalool, Eugenol, Methyleugenol, Trans-α-bergamotene, p-Cresol, 2, 6-di-tert-butyl, δ-Cadinene and (Z, E)-α-Farnesene (Nagai *et al*., 2011). Sifola and Barbieri (2006) reported that *O. basilicum* essential oil is constituted of phenylpropanoids, like eugenol, chavicol and its derivatives, and terpenoids; limonene, linalool and methyl cinnamate. Masi *et al*. (2006), studying nine different cultivars of *O. basilicum* commonly utilized, were able to identify five distinct chemotypes based on the main essential oil constituent; Iso-pinocamphone (35.1%) and carvone (39.7%) which were the predominant components of the essential oil in cultivated *O. basilicum*. This was also confirmed in another study by Almeida *et al*. (2010).

Several species of *Ocimum* have been reported to yield oils of diverse nature (Matasyoh *et al.,* 2007; Ogendo *et al.,* 2008). Other studies have also shown that the leaf extract of *Ocimum gratissimum* contain potential bioactive components of essential oils. These are made up of eugenol, citrol linalol, charvicol, thymol, gerianol, triterpenoids saponins and alkaloids (Atuboyedia *et al.*, 2010). It was also reported by Echeverrigaray *et al.* (2010) that monoterpenoids significantly reduce the hatching of eggs and mobility of J2 of *Meloidoyne* spp. Onifade (2007), in the study to find the effect of essential oils from five *Ocimum* sp. on the pathogenicity of *Pratylenchus brachyurus* (Godfrey) in tomato, reported that *in vitro* at 25-100 µg mL^{-1}, the oils of *O. gratissimum* and *O. basillicum* completely inhibited egg hatching and larval survival of nematodes after 24 hours. More study is still needed to explore the potential of essentials and other compunds involved in the nematode control in the two plant families Verbenaceae and Lamiaceae. The current work was therfore focused on investigating the potency of *Lippia kituensis* Vatke and *Ocimum gratissimum* L. on the management of root knot nematodes *Meloidogyne* spp. In this study we report results of a greenhouse pot experiment to evaluate management of root knot nematodes using fresh plant derived soil amendments with *Lippia kituensis* Vatke. and *Ocimum gratissimum* L. and their effects on tomato yield.

2. Materials and Methods

Experimental site

The study was conducted in two growing seasons at the Horticulture Research and Teaching Field, Egerton University, Kenya between July 2015 and May 2016. The site received a mean rainfall of 1012 mm with a mean day temperature of 22°C and night ranges of 5-10°C (Jaetzold *et al.,* 2012). The pot experiment was conducted in a polytunnel greenhouse measuring 8 m wide × 60 m length and 3 m height, covered with UV stabilized polythene sheet gauge 200 µm.

Plant materials

Leafy twigs of *L. kituensis* Vatke. and *O. gratissimum* L. were collected in the wild around Egerton University at flowering stage, when essential oil was

at its peak in the plants in the month of July 2015 (Fig. 1). These two shrubs flower all the year around visited by bees hovering over the flowers to collect nectar from scent produced by the plants. The materials were chopped into aggregates approx. 0.5 cm; the aggregates were incorporated in various proportions (0 -negative control, 200 g, 400 g and 800 g) in 10 kg of potted solarized forest soil, singly and in all possible combinations.

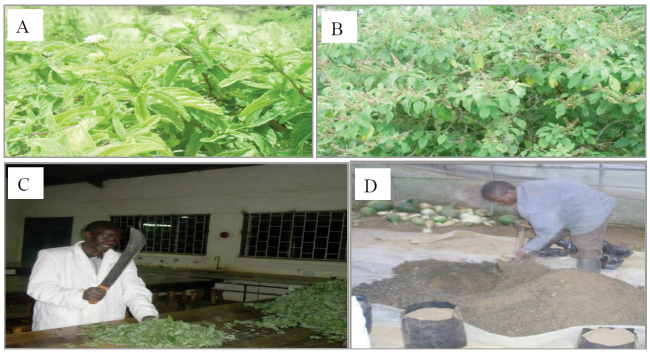

Fig. 1 - A. *Lippia kituensis* Vatke and B. *Ocimum gratissimum* L.; C. Chopping of the fresh *Ocimum gratissimum* plants into smaller pieces; D. Potting of the mixture (soil amendment process).

Crop establishment

The planting material used in the study was tomato seedlings 'Rio Grande' Rio Grande is a determinate tomato cultivar with a high yielding potential thus preferred by many farmers. This vigorous variety is well adapted to extreme temperatures. Due to the potential of heavy crops and to keep the fruit clean and easy to pick, it is recommended to support plants with stakes or cages. Seeds used to raise the tomato seedlings were obtained from Simlaws Seed Company in Nakuru (Kenya) and established in a nursery for 5 weeks before transplanting. Five weeks old tomato seedling were transferred to the substrates in the pots in the greenhouse to develop (Fig. 2). When plants reached a height of 30 cm, they were supported using sisal twines tied on a binding wire trellis at a height of 150 cm above the bed. Calcium ammonium nitrate (CAN, 26% N) was applied at the rate of 10 g per pot 21 days after transplanting (DAT) to maintain growth. Plants were pruned to maintain two stems per plant and watering was done continuously during the growing period with rates being adjusted according to plant growth phases. In the first 30 DAT, 2 l of water was applied per plant per day and thereafter, the rate was increased to 4 l to 42 DAT per day. From 43 DAT water supply was increased to 5 l per day as the plants developed, according to the work by Pires *et al*. (2011).

Fig. 2 - Transplants on treatment pots in the greenhouse.

Nematode augmentation, extraction and inoculum preparation

Nematodes were collected from a field previously grown with infested tomatoes and augmented on two weeks old potted tomato seedlings established in a greenhouse following the method of Siddiqui and Akhtar (2007). Specifically, Galls were extracted from the roots of infested tomatoes, chopped and mixed with the native soil. The mixture was added to pots planted with 2 week old tomato seedlings and the inoculum allowed to infest and multiply for 8 weeks.

After augmentation period, nematode egg masses were extracted from the heavily galled tomato roots by chopping the roots to lengths of 0.5 cm and macerating the tissues to release egg masses. These were placed in 15 cm diameter sieves of 1 mm pore size, lined with cross-layered tissue papers and incubated at 27°C to hatching in glass petri-dishes containing distilled water. After hatching, the second instar juveniles (J2) were transferred into 2 L conical flasks. Quantification of juveniles was done under a stereoscope with gridded petri dishes. Ten 1 ml replicate samples were drawn from the well mixed suspensions to establish the average number of juveniles per ml. The determined quantity was 20 juveniles per ml. Finally, the nematode inoculum suspension samples were adjusted to contain approx. 1000 juveniles in 50 ml of distilled water.

Nematode inoculation and determination of infestation parameters

The 50 ml J2s inoculum suspensions were added to pots containing the various plant biomass amended media planted with 28 day old tomato transplants. The inoculum was allowed to develop under normal tomato culture conditions in a polytunnel greenhouse. Destructive sampling of tomato plants to determine nematode infestation was conducted 100 DAT. Four plants from each replicate were sam-

pled at the peak of flowering.

Determination of macro nutrient from the tomato leaves

Nitrogen (Kjeldahl) and Phosphorus. Tomato leaves tissue were analyzed at 49 DAT for macro elements N, P, K, Mg and Ca. from the top of the plant, leaves were taken from the third and fourth leaflet per treatment. The sample materials were chopped into aggregates approx. 0.5 cm and oven dried at 70°C until constant weight. The oven dry plant samples were ground and wet digested by a sulfuric - percloric acid mixture as described by Cottenie *et al*. (1982). Nitrogen and phosphorus contents of vegetative samples were measured in the digesting extract according to the methods of AOAC (2012). Calcium and potassium content was determined in vegetative sample by ashing dry sample as described by Chapman and Pratt (1978) extract method.

Analysis of Potassium, Calcium and Magnesium. A substrate sample weighing 0.3 g was digested in digestion tubes using a digestion mixture comprising of HCl, HNO_3, HF and $H_3 BO_3$. The temperatures in the block was maintained at 360°C for two hours then samples cooled and transferred to 50 ml volumetric flasks and volume made to the mark. Calibration was done for each element using certified standards. Samples were analyzed using Atomic Absorption Spectrophotometer (AAS), Varian spectra AA10 AAS machine. The determination of these elements in the substrate was done using double acid method of extraction. AAS was used for estimation of these available elements in the tested substrate. This followed the procedure of Okalebo *et al.* (2002).

Experimental design and treatment layout

The experimental design used was a factorial embedded in a Randomized Complete Block Design and in total there were 17 treatments. There were three blocks and pots arranged in rows spaced at 0.6 m between and 0.4 m within the rows. There were 4 levels (0 g, 200 g, 400 g, and 800 g of organic amendments from each of the plant species, replicated 3 times. A negative control of solarized non-amended soil and a positive control of 0.3% w/w Azadirachtin, a farmer's standard commercially known organic based Neem extract were included. In total there were 17 treatment plots of 6 potted tomato plants in each block (Table 1).

Nematodes evaluation

Nematodes population. To determine the nematode population in the biomass amended pot soil treatments, second stage juveniles (J2) were extracted from 100 cm^3 composite sample of soil from each replicate, using the method described by Kimenju *et al.* (2010). Specifically, at 100 DAT, the soils from each of the four pots were sampled by taking 100 cm^3 of sample. The samples were placed in 9 cm diameter sieves with pore diameters of 1 mm lined with double layered tissue paper. The sieves were half immersed in metallic troughs containing 250 ml

Table 1 - Treatment combinations and description of soil amendment rates used in nematode management

Soil Amendments treatments (g/pot)	Description of treatments
0	0% w/w *Lippia kituensis* and 0% w/w *Ocimum gratissimum* (control)
200 OG	2% w/w *Ocimum gratissimum*
400 OG	4% w/w *Ocimum gratissimum*
800 OG	8% w/w *Ocimum gratissimum*
400 LK	4% w/w *Lippia kituensis*
200 LK +200 OG	2% w/w *Lippia kituensis* and 2% w/w *Ocimum gratissimum*
200 LK +400 OG	2% w/w *Lippia kituensis* and 4% w/w *Ocimum gratissimum*
200 LK +800G	2% w/w *Lippia kituensis* and 8% w/w *Ocimum gratissimum*
400 LK	4% w/w *Lippia kituensis*
400 LK+ 200 OG	4% w/w *Lippia kituensis* and 2% w/w *Ocimum gratissimum*
400 LK+ 400 OG	4% w/w *Lippia kituensis* and 4% w/w *Ocimum gratissimum*
400 LK+ 800 OG	4% w/w *Lippia kituensis* and 8% w/w *Ocimum gratissimum*
800 LK	8% w/w *Lippia kituensis*
800 LK +200 OG	8% w/w *Lippia kituensis* and 2% w/w *Ocimum gratissimum*
800 LK +400 OG	8% w/w *Lippia kituensis* and 4% w/w *Ocimum gratissimum*
800 LK +4 OG	8% w/w *Lippia kituensis* and 8% w/w *Ocimum gratissimum*
AZAD	Commercial (0.3% Azadirachtin) control

OG= *Ocimum gratissimum* L. (g/10 kg soil); LK = *Lippia kituensis* Vatke (g/10 kg soil), are soil organic amendments; 0 = no amendment; AZAD = positive control of commercially know pesticide Azadirachtin.

of distilled water to allow nematode migration into the water underneath for 24 hours. Nematode counts were determined in 10 replicate samples of 1 ml for each soil sample as previously described.

Gall number and galling index. For gall assessments, plants were gently uprooted and their roots thoroughly washed under tap water to remove all the adhering soil. Galling was determined by counts of galls size 1 mm diameter and above by a light microscope using the lowest objective lens x 4. Galled roots were spread in on plastic petri dish made of 1 cm^2 grids, numbered at the base. The galls were scored from each square to get the summation per plant. The galling index was scored on a scale of 1-10, where 0= no gall, 1= 1-50 galls, 2= 51-100 galls, 3= 101-150 galls, 4= 151-200 galls, 5= 201-250 galls, 6= 251-300 galls, 7= 301-350 galls, 8= 351-400, 9= 401-450 and 10= 451 and above (Kimenju *et al*., 2010). The scores were converted into numerical entries and their means worked out for analysis of variance.

Tomato growth evaluation

Number of leaves. Leaf count data was collected from 4 plants in each plot. Leaf count data collection was commenced 21 DAT and continued at intervals of 14 days up to 91 DAT. At each instance of data collection the mean number of leaves per plant from each replicate was computed. The mean number of leaves per treatment was determined by computing the means.

Plant height. Plant height data was collected from 4 plants in each plot. This started at 21 DAT and continued at 14 days interval up to 77 DAT. At each instance of data collection the mean height per plant from each replicate was computed. The mean height per treatment was determined by computing the means.

Root volume. Root volume data was determined by carefully removing the plants from the pot, shaking off the soil, and washed in running water on the trough at 100 DAT. Root volume was determined water displacement method in a one liter plastic measuring cylinder. The cylinder was filled to 500 ml mark, then the roots dipped carefully until the water just covered all the roots on the 4 plants used for root length determination and means computed to get the means.

Shoot and root dry weights. From the 4 plants used for root length and volume determination, shoots were separated from the roots at the collar. These were individually placed in kaki paper bags and dried in an oven at 70°C to constant weights. Both parts were weighed separately and means of the weights computed as above.

Physiological parameter

Stomatal conductance and chlorophyll content. Leaf stomatal conductance ($mmol \cdot m^{-2} \cdot s^{-1}$) was measured on four tomato tagged plants from each treatment. Using a steady state leaf porometer (SC-1, Decagon Devices, Pullman, WA), stomatal conductance was measured on a 2 weeks interval from 21 days after transplanting (DAT). Since tomato plants are hypostomatous, stomatal conductance was measured only on the abaxial leaf surface on 3 leaves on the upper parts of the plant and the average was computed. Leaf chlorophyll content was taken from the same leaves used for stomatal conductance. The instrument used was a chlorophyll content meter (CCM-200 plus, Opti-Sciences, Tyngsboro, MA) and measurement in chlorophyll concentration index units (CCIs), as an estimate of chlorophyll content on leaves.

Yield

Number of fruits per plant. Weekly piece meal harvesting of pink stage tomato fruits from the 4 tagged plants in each treatment was done. At each harvest, the number and weight of fruits were recorded for each treatment.

Marketable and Non-marketable fruit yield. Physiologically mature fruits (at pink stage) were harvested from the 4 tagged plants in each treatment. Harvesting was piece meal on weekly basis. At each harvest, fruits were sorted into marketable and non-marketable (kg/plant) separately and their weight determined and recorded. Fruit weight was taken from mature marketable fruits (at pink stage), harvested from the 4 tagged tomato plants from each treatment. These were weighed using a spring balance (ATZ, Shangai Precision and Scientific Instrument Co., Shangai, China) at each harvest and later summed up to give the total marketable weight (kg/plant).

Data analysis

Data for the two trials were pooled since there was no statistical difference between them. It was then subjected to analysis of variance (ANOVA) and means separated by the Tukey's HSD using The SAS statistical package version 9. The model fitted for this experiment was; $Y_{ijk} = \mu + \beta_i + \alpha_j + \gamma_k + \alpha\gamma_{jk} + \varepsilon_{ijk}$ where y_{ijkl} = tomato response, μ = overall mean, β_i = effect of the i^{th} block, α_j = effect of the j^{th} level of

Lippia kituensis Vatke ,γ_k = effect of the k^{th} level of *Ocimum gratissimum* L., $\alpha\gamma_{jk}$ = interaction effect of the j^{th} level of *Lippia kituensis* Vatke and k^{th} level of *Ocimum gratissimum* L. ε_{ijk} = random error component term which are normally and independently distributed about zero means with a common variance σ^2.

3. Results

Effect of soil organic amendment on nematode population, gall numbers and galling index

The different levels of amendments with *Lippia kituensis* Vatke and *Ocimum gratissimum* L. biomass and their combinations significantly (P<0.05) influenced nematode populations in the treatments (Table 2). Various rates of treatment reduced the juvenile populations when compared with the control. In single treatments of *Lippia* at 200 g, 400 g and 800 g, nematode numbers was reduced to 38.88 (25.9%), 19.29 (63.3%), and 20.58 (60.8%) respectively, compared to the non-amended treatments. *Ocimum* had similar trend with reduction to 47.38 (9.75%), 34.58 (34.1%) and 30.63 (41.7%), respectively (Table 2). However, the interactive effect of the two plant biomass from 400 g and above produced better nematode reduction than single treatments alone compared to soil.

Gall numbers and galling index were determined 100 DAT and were significantly P<0.05) influenced by organic amendments (Table 2). There was a general decrease of gall numbers in roots of tomato plants with increased levels of *Lippia* and *Ocimum* biomass in the potting soil. Application of *Lippia* singly at rates of 200 g, 400 g, and 800 g per pot reduced gall numbers to 190 (66.9%), 155 (73.0%) and 125.60 (78.1%) respectively compared to soil with *Ocimum* following the same trend with reduced galls numbers to 470.67 (18.1%), 422.5 (26.5%) and 175.3 (69.5%) respectively. Interactive effect of the two plant biomass above at the rates of 200 g was better reduction of gall numbers up to 25.17 galls (95.5%) and no significant differences (P<0.05) were evident among the various combinations.

The efficacy of the fresh plant biomass materials in managing nematode proliferation on tomato roots was also evident in the galling index scores from the various treatments. In general the gall index showed a reducing trend with increasing levels of *Lippia* and *Ocimum* biomass amendments. The interactive effect of the two plant species both at 800 g produced tomatoes with vigorous, fibrous root system with very few galls only observed under light microscope.

Table 2 - Effect of fresh organic amendments on nematode population, gall number and galling index

Amendments g/10 kg)	No. of nematodes (per 100 cm^3 soil)	No. of galls/plant	Galling index
0	52.50 a *	574.67 a	10.00 a
200 OG	47.38 b	470.67 b	10.00 a
400 OG	34.58 d	422.50 c	9.00 a
800 OG	30.63 ef	175.30 de	5.00 c
200 LK	38.88 c	190.00 de	7.30 b
400 LK	19.29 g	155.00 defgh	5.70 c
800 LK	20.58 g	125.60 defgh	3.00 de
200 LK + 200 OG	38.21 c	211.00 cd	4.67 c
200 LK + 400 OG	33.29 de	197.90 de	5.00 c
200 LK + 800 OG	28.33 f	123.40 defgh	3.67 cd
400 LK + 200 OG	17.50 gh	155.60 defgh	4.50 c
400 LK + 400 OG	12.25 i	130.17 defgh	3.00 de
400 LK + 800 OG	10.38 i	85.00 fgh	2.00 ef
800 LK + 200 OG	12.25 i	100.00 fgh	3.67 cd
800 LK + 400 OG	9.38 i	80.67 fgh	2.00 ef
800 LK + 800 OG	9.42 i	25.67 gh	1.67 ef
Azadirachtin	4.17 j	13.17 h	1.00 f

OG = *Ocimum gratissimum* L.; LK = *Lippia kituensis* Vatke; 0 = no amendment (soil).

* Means followed by the same letter series within a column are not significantly different according to Tukey's honestly significant difference (THSD) at P≤0.05.

Comparatively, those roots of plants grown in non-amended treatments (soil) had numerous galled roots which were less fibrous (Fig. 3).

Fig. 3 - The effect of *Lippia* and *Ocimum* on the tomato root system. A) LK+ OG at 800 g show fibrous root system, B) LK+OG at 200 g with fewer galls, and C) Non-amended soil high nematodes infestation.

Effect of fresh plant organic amendments on tissue of greenhouse tomato

The different levels of amendments of *Lippia* and *Ocimum* soil amendments had significant (P<0.05) influence on tomato plant tissue nutrients. The plant macro nutrient analyzed included N, P, K, Ca and Mg, which are the most essential element in tomato production. The interactive effect of the *Lippia* and *Ocimum* biomass were significantly higher in the tomato leaf tissues from both amendment rates above 400 g/10 kg of the substrate (Table 3). In single state, both plant biomass registered significantly higher N content at 800 g only.

Number of leaves

Tomato leaf numbers were significantly influenced by the use *Lippia* and *Ocimum* as soil organic amendments (Table 4). There were significantly (P<0.05) higher leaf number at 21 DAT in soil alone and 200g pots of both *Lippia* and *Ocimum* levels than 400g and above. However trend changed as from 35 DAT as from 35 DAT on 400 g of both species having the highest leaf numbers. At 49 DAT there were no significant difference (P<0.05 on treatment of both species from rates above as single or in combination 4 Non-amended soil had the least number of leave except for 21 DAT. Compared to the positive controls, Azadirachtin treated had relatively lower number leaves than 800 g of both *Lippia* and *Ocimum* combined, however this was higher than the un-amended soil.

Plant height

The result showed that the height of tomato plant was significantly (P<0.05) influenced by the *Lippia* and *Ocimum* levels (Table 5). Plants amended with 400 g of *Lippia* or *Ocimum* were taller than those of the lower levels of amendment and Azadairachtin. As in the leaf numbers plant height differences were observed 49 DAT were no difference were from each treatment up to the highest rate.

Root volume

The organic amendments significantly influenced the development of the total root volume of the tomatoes grown in the pots during the production seasons (Table 6). In root volume interactive effect

Table 3 - Effect of fresh plant organic amendments on tissue analysis of greenhouse tomato

Amendments (g/10 kg)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)
0	2.95 d *	0.23 e	2.82 g	0.68 l	0.28 f
200 OG	3.35 bcd	0.33 cd	3.12 ef	0.98 k	0.33 ef
400 OG	3.45 abc	0.30 cde	2.92 fg	1.18 i	0.32 ef
800 OG	3.35 bcd	0.32 cd	3.12 ef	1.68 f	0.36 de
200 LK	3.55 ab	0.33 cd	3.32 de	1.08 j	0.42 cd
400 LK	3.75 ab	0.43 b	3.42 cd	1.58 g	0.31 ef
800 LK	3.85 a	0.56 a	3.72 ab	2.08 b	0.49 abc
200 LK + 200 OG	3.35 bcd	0.34 cd	3.32 de	1.88 d	0.36 de
200 LK + 400 OG	3.75 ab	0.35 bc	3.72 ab	1.38 h	0.36 de
200 LK + 800 OG	3.55 ab	0.60 a	3.12 ef	1.78 e	0.38 de
400 LK + 200 OG	3.85 a	0.38 bc	3.52 bcd	1.88 d	0.35 def
400 LK + 400 OG	3.65 ab	0.37 bc	3.82 a	1.98 c	0.37 de
400 LK + 800 OG	3.65 ab	0.55 a	3.72 ab	2.38 a	0.42 cd
800 LK + 200 OG	3.65 ab	0.61 a	3.62 abc	2.38 a	0.46 bc
800 LK + 400 OG	3.55 ab	0.59 a	3.72 ab	2.08 b	0.52 ab
800 LK + 800 OG	3.65 ab	0.58 a	3.82 a	2.38 a	0.54 a
Azadirachtin	3.05 cd	0.26 de	2.92 fg	1.08 j	0.28 f

OG = *Ocimum gratissimum* L.; LK = *Lippia kituensis* Vatke; 0 = no amendment (soil).
* Means followed by the same letter series within a column are not significantly different according to Tukey's honestly significant difference (THSD) at P≤0.05.

Table 4 - Effect of fresh plant biomass on tomato leaf numbers

Amendments g/10 kg)	Leaf numbers				
	21 DAT	35 DAT	49 DAT	63 DAT	77 DAT
0	12.00 ab *	13.06 gh	20.83 cd	21.83 fg	22.67 k
200 OG	11.39 abc	12.61 h	20.94 bcd	22.67 ef	23.67 j
400 OG	10.28 cdef	14.61 cde	21.94 abcd	25.61 cd	26.20 fg
800 OG	0.28 cdef	14.78 cd	25.39 abc	27.33 abc	27.73 de
200 LK	12.21 a	13.28 fgh	21.83 abcd	26.61 c	27.66 de
400 LK	10.06 defg	13.5 efgh	16.94 d	20.11 g	24.67 i
800 LK	11.22 abc	14.28 cdef	24.89 abc	22.67 ef	27.20 e
200 LK + 200 OG	9.50 efgh	14.28 cdef	23.28 abc	26.83 bc	27.32 de
200 LK + 400 OG	11.22 abc	14.94 bcd	24.39 abc	25.79 cd	27.33 de
200 LK + 800 OG	10.94 bcd	15.22 abc	23.39 abc	26.72 c	27.27 e
400 LK + 200 OG	10.28 cdef	15.22 abc	24.11 abc	29.28 ab	29.53 ab
400 LK + 400 OG	9.28 fgh	14.06 defg	21.17 abcd	23.83 de	26.53 ef
400 LK + 800 OG	10.78 cd	16.21 a	23.50 abc	27.11 bc	29.60 a
800 LK + 200 OG	10.61 cde	15.39 abc	24.22 abc	26.18 cd	28.60 bc
800 LK + 400 OG	8.61 h	16.00 ab	24.56 abc	26.33 cd	29.73 a
800 LK + 800 OG	9.06 gh	16.33 a	27.39 a	29.83 a	29.90 a
Azadirachtin	12.33 a	13.56 efgh	23.89 abc	27.17 bc	28.13 cd

OG = *Ocimum gratissimum* L.; LK = *Lippia kituensis* Vatke; 0 = no amendment (soil). DAT = Days after transplanting.
* Means followed by the same letter series within a column are not significantly different according to Tukey's honestly significant difference (THSD) at P≤0.05.

Table 5 - Effect of fresh plant biomass from *Lippia kituensis* Vatke and *Ocimum gratissimum* L. on tomato plant height

Amendments g/10kg)	Plant height		
	49 DAT	63 DAT	77 DAT
0	48.78 fg *	91.56 f	91.56 f
200 OG	49.33 f	94.00 ef	94.00 ef
400 OG	71.72 ab	99.00 cde	99.00 cde
800 OG	70.11 abc	98.93 cde	98.93 cde
200 LK	58.22 def	98.60 cde	98.60 cde
400 LK	60.06 cde	102.80 abcd	102.80 abcd
800 LK	73.67 a	108.40 a	108.40 a
200 LK + 200 OG	66.61 abcd	97.47 de	97.47 de
200 LK + 400 OG	72.83 a	99.73 bcde	99.73 bcde
200 LK + 800 OG	67.06 abcd	97.80 de	97.80 de
400 LK + 200 OG	62.22 bcde	101.27 abcde	101.27 abcde
400 LK + 400 OG	72.83 a	105.73 abcd	105.73 abcd
400 LK + 800 OG	69.67 abc	108.27 ab	108.27 ab
800 LK + 200 OG	65.78 abcde	100.13 abcde	100.13 abcde
800 LK + 400 OG	66.50 abcd	106.53 abc	106.53 abc
800 LK + 800 OG	76.00 a	105.47 abcd	105.47 abcd
Azadirachtin	69.28 abc	88.40f	88.40 f

OG = *Ocimum gratissimum* L.; LK = *Lippia kituensis* Vatke; 0 = no amendment (soil). DAT= Days after transplanting.
* Means followed by the same letter series within a column are not significantly different according to Tukey's honestly significant difference (THSD) at P≤0.05.

was observe in the amendment at 400 g of both species with highest of 312.3 cm^3. Azadirachtin treated soils recorded relatively lower with root volume of 89.00 cm^3, which was not significantly different from the non-amended soil.

Root and shoot dry weight

As observed in the root volume, root and shoot dry weight showed effect by *Lippia* and *Ocimum* levels in the treatments for the two seasons in a similar trend (Table 6). Interactive effect of the two plant

Table 6 - Effect of fresh plant biomass on tomato leaf numbers

Amendments (g/10 kg)	Root volume (cm^{-3})	Root dry weight (g)	Shoot dry weight (g)
0	69.33 i *	24.17 h	46.12 j
200 OG	135.33 h	28.50 h	57.92 i
400 OG	84.6 7i	44.17 cdef	73.86 fg
800 OG	281.00 d	39.83 defg	69.95 fgh
200 LK	132.00 h	39.67 defg	71.42 fg
400 LK	249.67 e	39.58 efg	77.01 e
800 LK	282.33 cd	38.58 fg	86.56 c
200 LK + 200 OG	286.67 bcd	38.00 g	66.47 h
200 LK + 400 OG	229.33 e	43.00 cdefg	66.53 h
200 LK + 800 OG	279.67 d	46.50 bc	75.66 ef
400 LK + 200 OG	194.67 f	45.17 cde	86.10 c
400 LK + 400 OG	165.67 g	45.25 cd	96.68 b
400 LK + 800 OG	312.00 a	53.08 a	99.78 ab
800 LK + 200 OG	248.67 e	56.08 a	97.67 b
800 LK + 400 OG	310.00 ab	53.17 a	98.78 b
800 LK + 800 OG	306.00 abc	53.00 a	100.85 a
Azadirachtin	89.00 i	51.75 ab	86.23 cd

OG = *Ocimum gratissimum* L.; LK = *Lippia kituensis* Vatke; 0 = no amendment (soil).
* Means followed by the same letter series within a column are not significantly different according to Tukey's honestly significant difference (THSD) at P≤0.05.

were significantly higher weight as from 400 g and above. As was observed in the root volume, highest root dry weight of 53.1 g was not significantly different from Azadirachtin treated soils 51.7 g but significantly different from the control soil 24.1 g. similarly interactive effect of LK and OG registered 101 g, significantly higher than Azadirachtin treated soils 86.2 g and soil 46.1 g in LK 800 g combined with 800 g OG, Azadirachtin treated soils and soil alone respectively.

Effect of organic amendments on physiology response of tomatoes

Chlorophyll content and stomata conductance

Organic amendments levels influenced chlorophyll content in the tomato plant positively as shown in figure 4. For both season, interactive effect of both

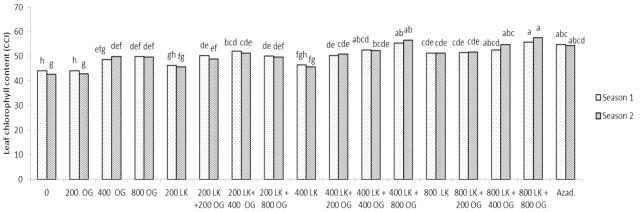

Fig. 4 - Effect of fresh plant organic amendments on tomato plant chlorophyll content taken after two week interval. Means followed by the same letter in a letter series within a column per season are not significantly different according to Tukey's honestly significant difference (THSD) at P≤0.05. OG =*Ocimum gratissimum* L. and LK = *Lippia kituensis* Vatke, are soil organic amendments in g/10 Kg soil, 0 = no amendment and AZAD = positive control of commercially know pesticide Azadirachtin.

plant species biomass were significantly higer than most of the single rates. Soil had the least chlorophyll content compared to the treated soils. The stomatal conductance of the leaves from the rates mentioned were affected in similar manner (Fig. 5). However Azadirachtin treated soil was not significantly different from highest combination of the biomass in stomatal conductance. Generally single treatments showed lower physiological process in the leaves.

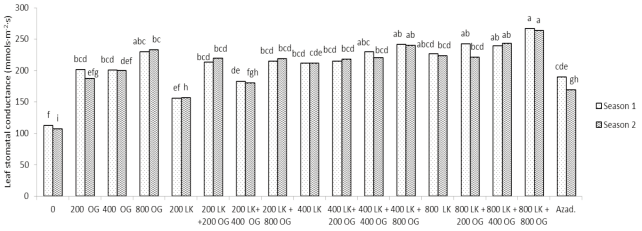

Fig. 5 - Effect of fresh plant organic amendments on tomato plant stomatal conductance taken after two week interval. Means followed by the same letter in a letter series within a column per season are not significantly different according to Tukey's honestly significant difference (THSD) at P ≤ 0.05. OG =*Ocimum gratissimum* L. and LK = *Lippia kituensis* Vatke, are Soil organic amendments in g/10Kg soil, 0= no amendment (soil), AZAD= Azadirachtin.

Number of fruits per plant

Lippia and *Ocimum*, significantly (P<0.05) influenced fruit number per plant (Fig. 6). Interactive effect of the two plants had mean of 62.72 fruits,

while non-amended control had 22.24 tomatoes per plant for season 1 and 2. Azadirachtin had 32.50 tomatoes per plant.

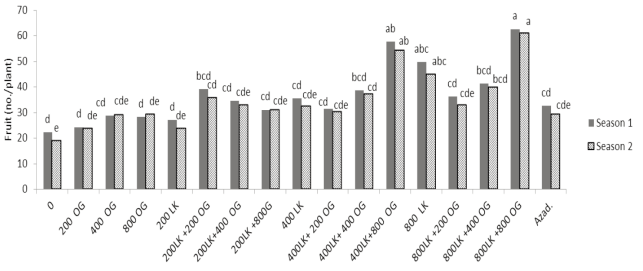

Fig. 6 - Effect of fresh plant biomass from the plant species Lippia kituensis and Ocimum gratissimum. on tomato plant fruit per plant. Means followed by the same letter in a letter series within a column per season are not significantly different according to Tukey's honestly significant difference (THSD) at P ≤ 0.05. OG =*Ocimum gratissimum* L. and LK = *Lippia kituensis* Vatke, are Soil organic amendments in g/10 Kg soil, 0 = no amendment and AZAD = positive.

Effect of fresh plant biomass on marketable yield and non-marketable yield

There was a general increase of marketable tomato fruits with the increase of the rates of plant organic amendment (Fig. 7A). The interaction between the especially above in *Lippia* or *Ocimum* 400 g produced tomato with higher t/ha than single treatments and soil media. Azadirachtin treated soil had better yield though significantly lower than those in 800 g of both species, which were rated as marketable.. Non-marketable showed a reverse trend on the fruit weight (Fig. 7). There was an opposite trend in the effect of the amendment from that of marketable. Any combination biomass lower than 400 g did not produce marketable but poor qualities fruits represented as blossom end rot, blotch ripening, puffiness, gold flex, car face, sunscald and very small size stony fruits, which rendered them non-marketable. (Fig. $7B_1$, $7B_2$, $7B_3$).

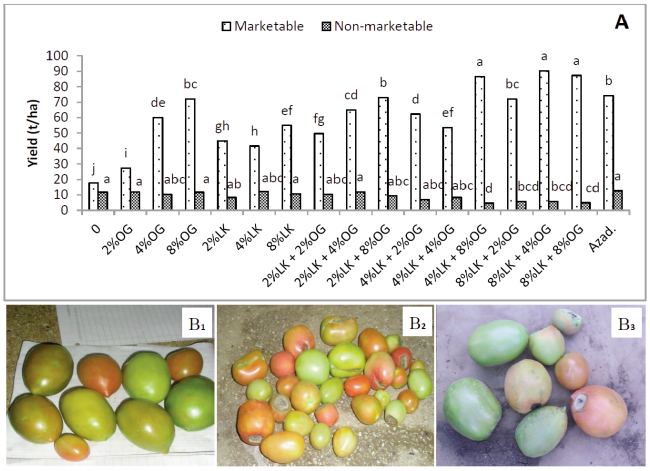

Fig. 7 - A. Effect of fresh plant biomass on marketable yield and fruit Quality. Means followed by the same letter in a letter series within a variable are not significantly different according to Tukey's honestly significant difference (THSD) at P≤0.05. OG =*Ocimum gratissimum* L. and LK = *Lippia kituensis* Vatke, are Soil organic amendments, 0 = no amendment and AZAD = Azadirachtin. (2% OG= 200 g OG, 4% OG= 400 g OG 8% OG= 800 g OG; 2% LK= 200g LK, 4% LK= 400 g LK, 8% LK= 800 g LK /10 Kg).
B. The picture shows difference in the quality of tomato at different rates of *Lippia* and *Ocimum* amendments B1= LK+OG at 800 g; B2= 0 LK+200 g OG; B3= Non-amended soil.

4. Discussion and Conclusions

The practice of adding organic matter to soil for management of soil pest and increase yield is as old as the agriculture (Akhtar and Alam, 1993) and this has been successfully explored to control some plant parasitic nematodes (Ferraz and Freitas, 2004; Lopes, 2011). This study revealed positive interactive effects of *Lippia kituensis* Vatke and *Ocimum gratissimum* L. as fresh biomass for the control of nematodes (*Meloidogyne* spp.) in greenhouse tomatoes. In overall, the results indicated effective nematode control in the tomato crop treated with the plant biomass compared to the control treatments where no amendments were applied. Additionally, it was generally observed that interactive effect of biomass treatments of the two species at the higher rates (400 g and 800 g) was more effective than the single treatments. In line with this study, Oka *et al*. (2007) reported sensitivity of plant-parasitic nematodes to plant derived amendments, however, they indicated that the effect varied with the nematodes species targeted and the rates applied. Our results further revealed that the second instar juveniles (J2) of *Meloidogyne* spp were more susceptible to higher rates of these treatments and effectively suppressed the nematodes in the media.

Several postulations on the mechanisms of action of these fresh biomass materials have been put forward. It has been reported that during the decomposition of these organic materials, volatile fatty acids, ammonia and hydrogen sulphide gases are released (McSorley, 2011) and these may enhance nematode control. Alternatively, other authors have explained the mechanisms of nematode population reduction by soil amendments with organic matter to involve

stimulation of antagonistic microorganisms, liberation of secondary volatile or nonvolatile phytochemicals with nematicidal properties (Lopes *et al*., 2011). As earlier reported by Chavarría-Carvajal and Rodríguez-Kábana (1998), the amendments may improve the growth of the plants and hence increase the tolerance and plant resistance to nematodes. In this study *L. kituensis* and *O*. *gratissimum* biomass additions to soil probably proved toxic to *Meloidogyne* spp. under greenhouse conditions, even at low rates of two species when combined at 200 g per pot of 10 kg of soil (Table 2). These results concur with the study of Lopes *et al.* (2011), who reported that soil amendment with the aerial portion of certain plant species has nematicidal properties. Similar results have been reported by Kagai *et al*. (2012), working with selected plant biofumigants in the management of plant parasitic nematodes in *Asclepias tubaerosa* L. In line with this study, Onifade (2007) earlier reported that essential oils from basil (*Ocimum basillicum*) had nematicidal effect on parasitic nematodes, especially *Meloidogyne* spp and *Pratylenchus penetrance* which is root lesion nematode. The second possible mechanisms for nematode suppression by these organic amendments could be direct inhibition or reduced infectivity of nematodes on the plant host. This may also be speculated that the use of *Lippia* and *Ocimum* as fresh soil organic amendment enhances antagonism in the soil mixes by increasing the abundance of other competing beneficial organisms, thus reduces the chances RKN survival. These results are in concurrence with a study by Claudius-Cole *et al*. (2010), which reported reduction of *Meloidogyne incognita* on cow pea *Vigna unguiculata* (L) Walp using plant extract. Besides, in agreeement with the present study Hasabo and Noweer (2005), earlier reported that the extract of *Ocimum* reduced nematode population on eggplant, and increased resultant fruit yields.

In the present study, different rates of amendments with *L. kituensis* Vatke and *O. gratissimum* L. biomass significantly influenced gall numbers and galling index in the treatments especially when the two plant biomass interacted (Table 2). This was demonstrated when galling index was drastically reduced. Various studies have shown similar observations in using organic amendments to control RKN. Breakdown of plant organic material releases nematicidal substances that may contribute to nematode control (Chen *et al*., 2000). Akhtar and Malik (2000) also reported that crops and weeds release biochemicals that counteract the activities of nematodes. This has also been confirmed by McSorley (2011) that nematicidal compounds released from decomposing materials can stimulate the natural enemies of nematodes and improving plant tolerance. In line with the present study, *Lippia* and *Ocimum* have been reported to yield essential oils of diverse nature (Atuboyedia *et al.*, 2010). Laboratory analysis of *Ocimum* yielded eugenol, citrol linalol, charvicol, thymol, gerianol, triterpenoids, saponins and alkaloids (Matasyoh *et al*., 2007; Ogendo *et al*., 2008). Based on the findings of the present investigation, it is plausible to suggest that these biomolecules extracted during decomposition of the plants biomasses helped to inhibit nematode activity in the amended soil, leading to low galling index. It also concur with observations by Onifade (2007) indicated that use of essential oils of *O. gratissimum* and *O. basillicum in vitro* at rates which completely inhibited egg hatching and larval survival of nematodes and this probably caused reduction of gall number.

From the present results on macro element analysis of the amended media, it is clear that besides acting as a nematicide for the management of RKN, *Lippia* and *Ocimum* also acted as plant nutrient source for tomato growth and yield. Mostly these element were significantly higher in the both amendment rates above 400g and above per 10 kg of the substrate (Table 3). Nitrogen, P, K Mg and Ca are essential macro element, important in entire plant growth and development. In particular, nitrogen is mostly required by plants to achieve high rates of growth and yield of tomato. The presence of these elements may promote physical and physiological changes in the plant and mostly related to photosynthesis, whereas Mg also plays a big role in chlorophyll structure (Taiz and Zieger, 2002). Nitrogen is a critical macronutrient influencing processes growth and development directly on source-sink relations, altering the distribution of assimilates between the vegetative and the reproductive part resulting into yield (Zuba *et al*., 2011). Phosphorus is for root development, flower initiation, seed and fruit development. Unlike N and P, K does not form any vital organic compounds in the plant, however, its presence is vital for plant growth, being known to be an enzyme activator that promotes metabolism (Silva and Uchida, 2000).

The effect of *Lippia* and *Ocimum* rates and their interaction on vegetative phase, increased leave number and height was revealed in this study (Table 4). With increased rates of organic amendment in the combination, it is probable that NPK levels in the soil

may also enhance growth, leading to the increase in leaf number. Leaf number is a function of N in plant (Otieno *et al.,* 2019) and this is very key for the higher number of leave observed at the rates of 800 g *Lippia* and 800 g of *Ocimum* combined. At DAT 21 there were more leaves in the non-amended soils compared to amended soil in seasons 1 and 2 (Table 4). This was probably due to the loss of nutrient especially N from the decomposing fresh organic amendments by microorganisms involved. The microorganisms involved in the decomposition possibly out-competed the tomatoes in the used the N available for the plant and this may have led to reduction in growth rate. However, this trend was changed as from 35 DAT upwards indicating that both *Lippia* and *Ocimum* had started releasing nutrient from decomposition process for tomato use. This is in conformity with observation made by Pakeerathan *et al.* (2009) in the management of *Meloidogyne incognita* using different green leaf manures on tomato under field conditions, where N contributed more toward the vegetative components (leaves and stems) of the plant than reproductive components.

The height of tomato was also influenced by the higher rates *Lippia* and *Ocimum* (Table 5) and probably this was a function of K in the organic amendments rates applied as observed in table 3. This is in consistence with El-Nemr *et al.* (2012) who reported Potassium (K) concentration as among the plant macronutrients that affected these growth parameters. In another study, Faruk *et al.* (2011) reported similar observation on the effect of poultry organic amendment on root knot nematode management and its influence on the height of greenhouse grown tomatoes.

The ability of plants to obtain water and mineral nutrients from the soil is related to their capacity to develop extensive roots and root hairs (Taiz and Zieger, 2002). As in Table 6, there was a significant increase in root volume with increased rates of *Lippia* and *Ocimum* especially when the two species were combined above 400 g, compared single rates and the controls. For root growth and development, organic amendments in the soil has been known to increase the bulk density of the growing media (Otieno *et al*., 2020), giving room for root system to explore wider range of the soil environment for more nutrients (Faruk *et al*., 2011; Otieno *et al.,* 2019). At lower amendment level, fewer root were observed, resulting in low root volume. In contrary, roots produced from 800 g of *Lippia* and *Ocimum* produced higher volume of fibrous roots. From this study it may be speculated that the root system of the tomatoes from highly amended media probably were affected in two ways; either by enhancing soil structure in favour of the roots growth (Otieno *et al.,* 2019), or reduction of nematodes population in the soil or both. Increasing amendments to the soil may alter many factors that affect root development in the rhizosphere. These include soil structure, particle aggregation, pH, salinity, level of Carbon dioxide, Oxygen and other chemicals (Akhtar and Malik, 2000). In this way, this probably increased the roots' ability to increase in dry weight and subsequently shoot dry weight. Similarly, the current result concurs Yadessa *et al*. (2010), whose findings showed that 10% of FYM produced significantly higher shoot and root dry weight compared to non-amended.

Leaf chlorophyll content (CCL) and stomatal conductance responded positively to increasing rates of these organic amendments. At 49 DAT, it was marked with maximum physiological processes in the crop (Figs. 4 and 5), indicating higher formation of chlorophyll content on the tomato crop at higher rates of *Lippia* and *Ocimum.* Nitrogen is an essential nutrient for normal growth and development of a plant as it is an integral part of the chlorophyll molecule (Kitonga-Mwanza, 2011), together with Mg; the principle site of light absorption necessary for photosynthesis. Stomatal conductance on the other hand was similarly influenced by both *Lippia* and *Ocimum* rates in the organic amendments (Fig. 4). Mostly K is involved in stomata closing and opening, therefore when galls have interfered with root system, the stomatal function may have been negatively affected at lower rate of amendments showing lower rate of stomatal conductance. This suggests that apart from the influence of K in the organic amendments, nematodes also played a part by the interference of the plant root system (Mai and Mullin, 1996), reducing the flow of water and minerals upward the plant.

From the current study, amendments rates influenced yield differently among the individual rates (Fig. 6). Highest yield in terms of fruit numbers registered per plant was observed in those with 400 g and above in rates of *Lippia* and *Ocimum* amendments, indicating that the production of fruits was probably from primary plant nutrients as reflected in tomato tissue analysis (Table 3). This observation is in agreement with that made by Walker (2007), where, the effect of organic amendments, fertilizers and fenamiphos was reported on reduction of parasitic and free- living nematodes as well as increased yield

of tomato. A number of authors have reported that adequate K nutrition is linked with increased yields (Kanai *et al.,* 2007; Afzal *et al*., 2015), which further confirm the current findings plant derived organic amendments and their impacts on tomato production.

Finally, the marketable yield of tomato is basically dependent on regular nutrient and moisture availability in growing media for plant use. Moisture in particular is essential for nutrient movement, whereas its irregular flow in the plant system may cause blossom end rot of (BER) in tomato. This scenario is more pronounced irregular Calcium mobility in both growing media and the plant system. As for the current study, application of both *Lippia* and *Ocimum* at 800 g level per 10 kg pot seemed to have increased water holding capacity of the amended soil since organic matter enhances the moisture availability in the media leading to more nutrient availabilty hence higher marketable yield. In concurrence to the present study, Akhtar and Malik (2000) reported higher marketable yield following the application of organic matter to the soil. The beneficial effects of organic amendments are generally assumed to be due to the provision to the crops, extra nutrients. The current study therefore emphasizes that yield obtained from high level amendment of *Lippia* and *Ocimum* were generally higher in marketable quality compared to non-amended soils. Conclusively, both plant organic amendments played an important role as both biopesticides and organic soil fertility for crop growth and development.

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Mechanical rubbing of tomato internode influence stem growth, improve tensile strength but negatively impact flavonoid levels

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Key words: Lignin deposition, *Lycopersicon esculentum*, mechanical induced stress, phenolic compounds, tomato.

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JMN conceived the experiments and SS conducted the experiments. JMN and SS contributed to the analysis, interpretation and writing of manuscript.

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Abstract: Agricultural crops are exposed to different environmental stress factors on a daily basis. Mechanical induced stress (MIS) caused due to contact rubbing, bending, transplantation and spraying of water results in altered growth in plants (Thigmomorphogenesis). The present study was conducted by inducing mechanical stress (gentle rubbing) on the third internode of tomato (*Lycopersicon esculentum* Mill.) by pressing with thumb and index finger (30 sec) for 14 consecutive days. At the end of the stress period, marked morphological differences included significant reduction in plant height and decreased internodal length of the rubbed third internode as well as the neighboring fourth internode. Histochemical staining of the stem cross section of stressed plants showed intense color indicating lignin deposition. Study of biochemical response post internode stress showed an increase in total phenol content but lower flavonoid contents. Stress induction also resulted in modification of biomechanical characteristics like tensile strength, elastic modulus and breaking force. Studies on the effect of mechanical perturbations in plants has gained attention because of its implication in fundamental processes of organogenesis/morphogenesis and their potential as an innovative means of controlling plant growth.

1. Introduction

In the natural habitat, plants are challenged by various biotic and abiotic stress factors. Mechanical Induced Stress (MIS) is one such form of stress that occurs inevitably in plants on a daily basis. MIS is a term used in cases of either a damage or physical injury due to bending, shaking and rubbing experienced by plants as a result of wind exposure, rain, animal movement and agricultural practices (Biddington, 1986). Being sessile, plants do not possess the luxury of relocating from the stressful source and are persistently challenged by these unfavorable environmental conditions. Plants are also known to sense the force of stimuli that can range from subtle effects like touching or rubbing on leaves to more intense

ones like the damage caused by herbivores (Chehab *et al*., 2009). Morphogenic responses due to rubbing or touch are termed Thigmomorphogenesis (Jaffe, 1973). Among the most visible morphological thigmomorphogenesis response common to many plant species includes decrease in shoot elongation, coupled with increased radial dimension of cortex cell (Telewski and Jaffe, 1986; Braam, 2005; Potocka and Szymanowska-Pulka, 2018). Plant cell wall is the first barrier against external mechanical forces. Lignin is one of the main structural components of the plant cell wall and regulation of lignin metabolism is of prime importance to plant growth (Zheng *et al*., 2017). Apart from lignin, flavonoids are among the most crucial phenolic compounds involved in various protective roles in plants, such as attractants, feeding deterrents and even, oxidative stress protection (Treutter, 2005; Mierziak *et al*., 2014). While, stress induced biochemical changes in lignin are known, effect of mechanical stimulation on flavonoid levels are scarce.

Regulation of plant growth to achieve essential agronomic traits such as crop yield and plant height, are important prerequisites for agricultural industries. Marketability of many important ornamental and horticultural crops increases with compact height and a strong stem as these facilitates packaging and also, transportation of post-harvest produce (Babalar *et al*., 2016; Bornke and Rochsch, 2018). The knowledge on force response of stem structures is critical as it incorporates approaches to test biomechanical properties involving tensile strength and elastic modulus. These tests besides having economic implication, is also an immense tool for crop improvement (Shah *et al*., 2017). Tomato, (*Lycopersicon esculentum* Mill.) is one of the most cultivated vegetable crops belonging to *Solanaceae* family (Schwarz *et al.*, 2014). Global tomato production accounts for 170 million tonnes, comprising 75% fresh and 25% processed material for industrial uses (Costa and Heuvelink, 2018). Due to its short duration and relative ease of cultivation, tomato production has become one of the principal horticultural industries (Costa and Heuvelink, 2018).

In this study, we experimentally determined whether stress on the internode by rubbing can lead to thigmomorphogenetic effects, leading to mechanical stress resistance in tomato. It is hypothesized that lignin accumulation due to mechanical perturbations positively regulates higher tensile strength in plant stem. Therefore, the purpose of this study was to establish physiological effects of mechanical stimulation of the 3rd internode on tomato stem growth characteristics and consequently, its mechanical properties in plants.

2. Materials and Methods

Plant material and growth condition

Seeds of tomato (*Solanum lycopersicum* Mill.) belonging to a semi determinate variety (*Solanum lycopersicum* Mill. cv. Arka Vikas) were obtained from Indian Institute of Horticultural Research (IIHR, Bengaluru, India). Germinated seedlings were transplanted to fresh pots (one plant per pot) in green house (12°56‘7” N, 77°35‘3” E) and maintained in moist soil under natural light condition (14 hrs of light and 10 hrs of dark) at 27-28°C. Relative humidity was maintained around 61-70% in the green house. All the experiments were conducted between 10.00 AM - 2.00 PM to avoid early and late diurnal response.

Application of mechanical stimulation

Mechanical stress stimulus was applied to five week old plants, henceforth referred to as internode stress. Internode stress was applied by rubbing the 3rd internode for 14 days, using thumb and index finger for 30s (Depege *et al*., 1997). Plant growth parameters like height and internode length were measured after the application of internode stress for 14 days. The mean total height and internodal length (3rd and 4th internode) of 20 plants were recorded and compared with control plants.

Total phenolic compound content

Total phenolic content in leaves of plants after application of stress were determined by Folin-Ciocalteu method as described by Marinova *et al*. (2005). About 1 gram of fresh leaf was collected by excising the third leaf through random selection from the 20 pots of both control and stressed plants. Evaluation of phenol content was made for each of the independent triplicates (n=3) from 1g of pooled leaf samples for both control and treated plants. Each sample was macerated in 25 ml of methanol for 24 hr with occasional shaking. 250 µl of the extracted solution was mixed with 750 µl of methanol and 1mL of Folin-Ciocalteu reagent was added. After 5 min, 1 mL of Na_2CO_3 (20%) was mixed to the solution. After 30 min of incubation in dark, absorbance was measured at 765 nm using a UV-Vis Spectrophotometer (Shimadzu Inc. Japan) against a blank sample. A stan-

dard Gallic acid curve was constructed by preparing dilutions of a standard solution of Gallic acid. Total phenol was calculated and was expressed as Gallic Acid Equivalents per gram of fresh weight (GAE g^{-1} of FW).

Total flavonoid content

Aluminium chloride calorimetric method was followed for the determination of the total flavonoid content in leaf samples based on the methodology described by Dewanto *et al*. (2002). About 1 gram of fresh leaf was collected by excising the third leaf through random selection from the 20 pots of both control and stressed plants. Evaluation of flavonoid content was made for each of the independent triplicates (n=3) from 1 g of pooled leaf samples for both control and treated plants. Each sample was macerated with 70% methanol and kept for 24 hours with occasional shaking. The extracted sample solution (250 µL) was separately mixed with 75 µL of 5% $NaNO_2$ followed by incubation for 5 min. After incubation, 10% $AlCl_3$ (150 µL) was added followed by 500 µL of 1 M Sodium hydroxide. Total volume was made up to 2.5 ml using distilled water. After the incubation period for 30 min, absorbance was measured at 510 nm against a blank using UV-Vis spectrophotometer (Shimadzu Inc. Japan). Quercetin standard curve was constructed by preparing dilutions of a standard solution of Quercetin for the quantification of total flavonoids. The total flavonoid was calculated and was expressed as quercetin equivalent per gram of fresh weight (QE g^{-1} of FW) (Lin and Tang, 2007).

Lignin staining and quantification analysis

To evaluate how MIS affected lignin deposition, histochemical analysis of the stem sections of control and stressed plants was performed using wiesner reagent. The amount of 2% of fresh phloroglucinol staining solution for lignin staining was prepared in 95% ethanol. Fresh stem sections from the neighbouring 4^{th} internode were immobilized on a sliced potato surface. Multiple homogeneous thin freehand sections were obtained by drawing a sharp razor blade with smooth stokes repeatedly to ensure uniformity. The sections were further checked by stereomicroscope in order to remove thick and also damaged sections. The most uniform sections were picked carefully and transferred to petri plates containing water. Uniform sections of control and treated plants were transferred to petriplates and immersed in phloroglucinol stain. After 24 hours, sections were transferred to a clean glass slide, to which a drop of concentrated HCl was added (Phloroglucinol-HCl stain). The slides were then gently warmed over a Bunsen burner for 20 seconds for HCl evaporation. A drop of glycerol was added to these sections after which a cover slide was placed over it gently. The slides were then observed under a microscope Labomed Vision 2000 (Inc. India) at 10X magnification (Mitra and Loque, 2014).

Lignin quantification analysis was conducted through ImageJ software (ver 1.52p). An eyepiece graticule (scale bar) was used to set the scale measurements for the sections of control and treatment. These images were further processed through a process of spatial resolution information in ImageJ using the calibration set by the graticule image. To start with the quantification analysis, images were duplicated. One of the images was converted from 8 bits to grayscale. Following this action, pixel information pertaining to the pixel colour, gets converted into brightness measurement. By selecting the command: image>adjust>threshold, images to be quantified was highlighted in pixels within the threshold range specified. Next, by choosing the command: analyse>set measurement, the area and limit to threshold was selected. This enabled to measure within the threshold pixel range for control and treated images. Finally, the function, analyze-measure was used to read the area covered with stain. Percentage of stained area was calculated by: Stained area/Total area X 100 (Beziat *et al.,* 2016).

Determination of tensile strength

Determination of biomechanical stem properties was done after 14 days of stress application. Plants of 10 cm length were defoliated after the experiment and five stem segments devoid of leaves and roots were randomly chosen for analysis from stressed and control plants. These stem segments were then kept in an oven (40-50°C) for approximately 1 hour to rid of excessive water content. They were then clamped in the Universal testing machine (MTS-Mechatronics, Inc, Ichalkaranji, India). The following biomechanical traits were determined (Shah *et al*., 2017):

(1) Tensile strength (MPa) is a measure of the maximum stress that the material can withstand without being elongated or stretched.

(2) Elastic modulus (MPa) represents a ratio of stress applied and strain, the material exhibits (degree to which the material resists deformation in response to an applied force).

(3) Breaking force (N) measures the maximum force that the material can bear before its experiences mechanical stress failure.

Statistical analysis

Statistical analysis for the results obtained were carried out using Student's *t* test with level of significance set at P<0.05. Coefficient of variation (CV) is a useful measure to determine dispersion in a variable and can be extremely informative to study variation in different phenotypic traits in a study. Coefficient of variation for the morphological traits obtained was calculated as follows:

(Standard deviation/Average) x 100.

Pearson correlation coefficient was performed to study the level of association between two variables, pair-wise. All these analysis was performed using GraphPad Prism (version.8).

3. Results

Effect of internode stress on plant height and internodal length of tomato

Application of mild mechanical stimuli in the internodes of treated plants resulted in reduction in plant height whereas control plants showed normal growth characteristics (Fig. 1). Internode stress led to 27.4% reduction of total plant height when compared to control plants, a significant reduction at P<0.0001 (Fig. 2 a). Effect of internode stress affected the elongation of 3rd internode, the site of stress application. Interestingly, internode stress also resulted in significant difference in elongation of the neighbouring 4th internode. Results from the present finding showed that elongation of stressed internode was inhibited by 47% and 44% for 3rd and 4th internode respectively at P<0.0001 in comparison to control plants that were not stressed (Fig. 2 b).

Coefficient of variation (CV) studies

The results presented in Table 1 revealed that the mean plant height in control plants was 27.5 cm with a range between 25.2-30.5 cm. In contrast, treatments resulted in mean plant height of 20.01 with range between 18.0-22.8 cm. In case of treated plants, the highest CV was observed for 3rd internode (16.64%), the site of mechanical stimuli followed by the 4th internode (14.8%). In comparison, in control conditions, coefficient of variation (CV) of 5-7% was observed for the traits measured, confirming lower variation within population. Clearly, internode stress

Fig. 1 - *Lycopersicon esculentum* Mill cv. Arka vikas after 14 days of application of mechanical stimuli by rubbing the 3rd internode. Left, control plants and right, treated plants.

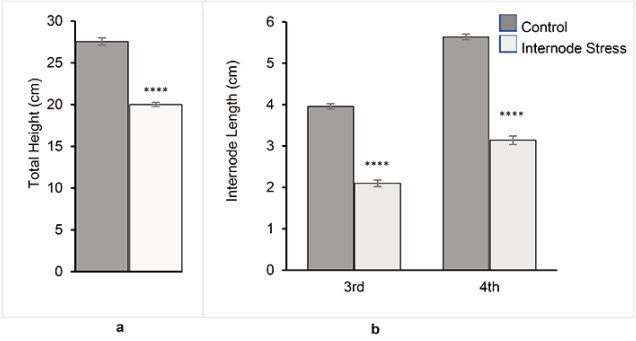

Fig. 2 - Total height of control and internode stressed plants (a) and length of third and fourth internode of control and internode stressed plants (b). The vertical bars indicate standard errors. The values correspond to the mean ± SE, where n=20. Statistical analysis was performed by Student's t-test for total height and internode length. Asterisks indicate mean values significantly differ at P<0.05 (**** indicate P <0.0001).

Table 1 - Range of group means, mean values, standard deviation, standard error mean and coefficient of variation of internode length and total height of control and treated plants

Traits	Control					Treatment				
	Range	Mean	SDV	SEM	CV (%)	Range	Mean	SDV	SEM	CV (%)
3rd Internode	3.6-4.5	3.9	0.287	0.064	7.3	1.5-2.6	2.1	0.349	0.078	16.64
4th Internode	5.2-6.3	5.6	0.298	0.066	5.3	2.5-3.8	3.1	0.46	0.103	14.8
Total Height	25.2-30.5	27.5	1.9	0.429	6.9	18-22.8	20.01	1.097	0.245	5.48

* Values in the table mentioned for n=20. Descriptive statistics for control and treated plants SDV= Standard deviation; SEM= Standard error of mean; CV= Coefficient of variation.

lead to higher variability for the 3rd and 4th internode than in normal conditions as indicated by these results.

Effect of internode stress on total phenol and flavonoid content

Mechanical stimuli resulted in an increase of 25% total phenolic content in leaves, when compared to control plants, a significant increase at P = 0.0001 (Fig. 3 a). In contrast, flavonoid content in leaves of treated plants showed a reduction of 18% in comparison to control plants, a significant decrease at P = 0.0013 (Fig. 3 b).

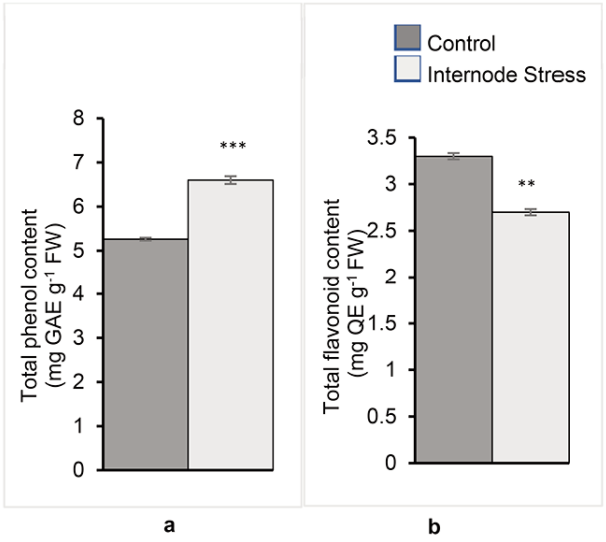

Fig. 3 - Total phenol content expressed as mg of gallic acid equivalent per gram of fresh weight (GAE g^{-1} FW) after 48hrs of internode stress (a) and total flavonoid content expressed as mg of Quercetin Equivalent per gram of fresh weight (QE g^{-1} FW) after 48hrs of internode stress (b). Standard error is indicated by vertical bars. The values correspond to the Mean ± SE, where n=3. Statistical analysis was performed by Student's t test. Asterisks indicate Mean values significantly differ from control at P <0.05 (** indicate P = 0.0013 and *** indicate P = 0.0001).

Effect of internode stress on lignin deposition and tensile strength

Histochemical analysis by staining revealed that the intensity of colour was visibly lower in control plants (Fig. 4 a). On the other hand, analysis revealed intense colouration (red/magenta) in stressed plants (4th internode) indicating increased lignin deposition in xylem bundles and confirming alteration in lignin deposition in the plants with internode stress (Fig. 4 b). Lignin quantification through ImageJ analysis revealed a 45% enhancement in lignified area for sections from treated plants. On the other hand, sections from control samples covered only 22% of lignified area (Fig. 4 c).

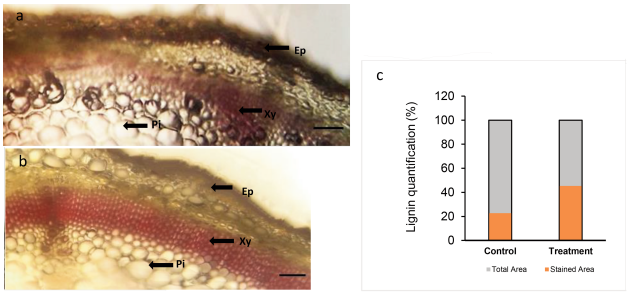

Fig. 4 - Microscopic view of stained cross sections after 14 days of rubbing of the third internode for control (a) internode stressed (b) and graph showing quantification of lignin analysis by ImageJ software (c). Ep-epidermis, Xy- xylem (lignin deposition) and Pi-pith. Scale bar = 100 µm.

Application of mechanical stimuli also led to increased mechanical strength as indicated by the various mechanical properties shown in figure 5. The mean tensile strength recorded a significant increase of 113% in treated plants, when compared to control plants at P<0.05 (Fig. 5 a). Other mechanical properties including elastic modulus, and breaking force were also significantly higher when compared to the control plants. The elastic modulus displayed by treated plants observed was 658.4 MPa, a highly significant increase (P<0.0001) compared to control plants (Fig. 5 b). The mean breaking force for treated plants recorded was 21.13 N, in comparison to control plants, which was lower (9.8 N), a significant difference at P= 0.0007 (Fig. 5 c).

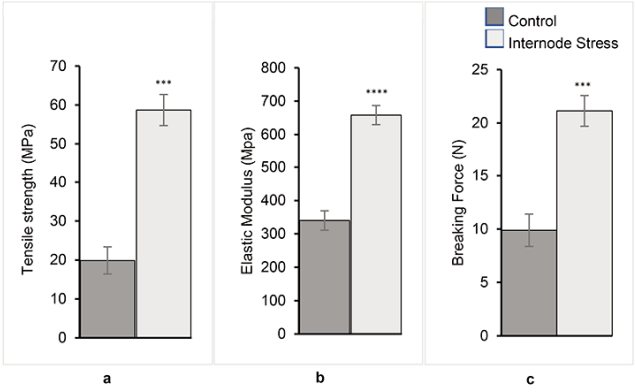

Fig. 5 - Biomechanical properties of the stem measured through tensile test: tensile strength (a), elastic modulus (b) and breaking force (c). Standard errors are indicated by vertical bars. The values correspond to the Mean ± SE, where n=5. Asterisks indicate Mean values significantly differ at P<0.05 (*** indicate P=0.0007 and **** indicate P<0.0001).

Correlation studies among biomechanical trait

The correlation coefficient among tensile strength, breaking force and plant height for both control and treated plants are reported in Table 2. Plants treated with internode stress showed a highly significant positive correlation (R = 0.999) among the two important mechanical properties for stem, tensile strength and breaking force. However, the correlation for these traits in control plants remained non-significant. The correlation of total height of plant with respect to tensile strength and breaking force demonstrated a significant negative correlation for both treatment and control plants. Plants treated with internode stress showed a slightly higher negative correlation (R= -0.97) than control plants (R= -0.91) between tensile strength and total height. A scatter plot of these associated traits clearly highlighted a negative slope of correlation after exposure to mechanical stimuli (Fig. 6).

Table 2 - Linear correlation coefficient between different biomechanical characters in control and treated plants. Correlation of morphological trait with biomechanical characteristics

Traits	Total plant height	Breaking force
Control		
Tensile Strength	-0.91 *	0.7 NS
Total Plant Height		-0.94 *
Treated plants		
Tensile Strength	-0.97 **	0.999 ****
Total Plant Height		-0.95 **

Asterisk indicates statistical significance, at P=0.12(NS), 0.033(*), 0.002(**) and <0.0001(****), NS= non significance difference.

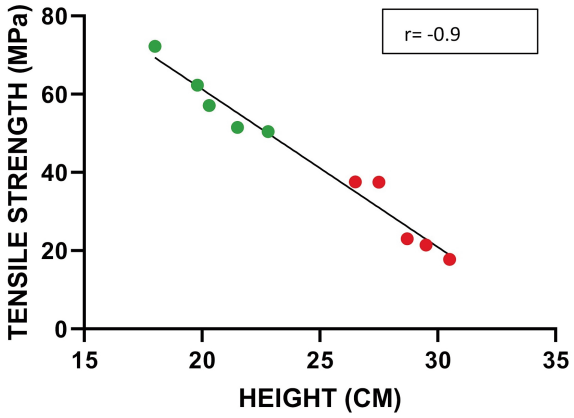

Fig. 6 - Scatter plots of tensile strength and total height measured on treated plants (green) and control plants (red). The scatter plot indicates a significant negative correlation between two variables with a correlation coefficient of r=-0.9, (P<0.0001).

4. Discussion and Conclusions

In response to mechanical stimuli, plants respond by altering their growth rate and morphology (Chehab *et al*., 2009). In this communication, application of internode stress was shown to be effective in reducing the overall height of the plant. Effect of brushing in reducing leaf size and stem elongation has been demonstrated in *Lycopersicon esculentum* (Mill.) (Johjima *et al*., 1992). In *Arabidopsis thaliana* (Linn.), inhibition of stem elongation in the inflorescence was also shown to be prominently affected due to mechanical perturbation (Victor and Rowe, 2011). A higher coefficient of variation in treated plants shown in this study demonstrated that the most relevant effect of internode stress treatment was to bring growth alteration by causing reduction in internode elongation for both the 3rd and the 4th internode. In comparison to the treatment, coefficient of variation in control plants was lower, revealing insignificant random variation within a phenotypic trait. Hence, reduction in internodal length clearly indicates that mechanical stress response is a generalized response affecting both the rubbed internode as well as neighboring young internode. Plants do not possess specialized cells to mount a response to Mechanical Induced Stimuli (MIS) in nature (Börnke and Rocksch, 2018). Individual cells in the vicinity of local tissue that is mechanically stimulated may possess the property of mechanoperception and to transduce this signal to an unperturbed distal tissue either by hormonal or electrical signals (Erner *et al*., 1980). Depege *et al*., (1997) suggested that the transduction of the signal was electrical occurring through the involvement of intercellular Ca^{2+} modulated by calmodulin gene expression. Moulia *et al*. (2015) further proposed that local mechanosensing and the origin of ionic currents can be explained through the participation of mechanosensitive ionic channels or stretch-activated channels (SAC).

Under our experimental conditions, while the phenolic contents increased, flavonoid content was reduced by application of mechanical stress. In plants, the phenylpropanoid metabolism and also the lignin biosynthetic pathway are stimulated by environmental stress adaptation (Petersen *et al.,* 1999). Saidi *et al*. (2009) reported an increase of all the major enzymes involved in the phenylpropanoid pathway with mechanical stimulation in tomato. Consequently, an increase in phenolic content and

enrichment of lignin observed in this study is consistent with these reports. However, biosynthesis of lignin pathway and flavonoid are highly co-regulated in plants, where repression of one pathway, redirects the metabolites into the other pathway (Besseau *et al*., 2007). In the phenylpropanoid pathway, *p*-coumaroyl CoA is the branch point in the metabolic route leading towards biosynthesis of either one of them (Yeh *et al*., 2014). In plants, accumulation of flavonoid is affected as the common flavonoid-lignin pathway is diverted towards utilization of support mechanism for biosynthesis of lignin (Besseau *et al*., 2007). A reduction in flavonoid content observed in this study therefore, points to the proposition that lignin and flavonoid may be competing for the same substrates leading to a decrease in flavonoid synthesis.

MIS increased stem resistance to tensile forces in this study. The modification in these parameters is relatively fast as the experiment lasted for duration of only 14 days. In plants, important biomechanical traits for instance, higher resistance to tensile and breaking forces leads to the acquisition of a hardened phenotype (Schoelynck *et al*., 2015; Shah *et al*., 2017). Cell wall rigidification due to accelerated lignification was shown to result in reduced internodal elongation after mechanical stress application in tomato (Saidi *et al*., 2009). Further, reinforcement of cellulose microfibrils by lignins was also reported to increase resistance to tensile forces (Genet *et al*., 2005). This unique thigmomorphogenetic response in plants is crucial to withstand repeated external stress and mechanical forces (Jaffe *et al*., 1984; Biddington, 1986). Moreover, plant height was shown to be negatively correlated with tensile strength and breaking force in this study. Plant lodging, an important trait observed in plants is shown to occur as a consequence of permanent displacement of plant stem by stem buckling (Kendall *et al*., 2017). Reduction in plant height and internodal length has been shown to improve lodging tolerance in crops, an important trait for crop improvement (Peng *et al*., 2014). Notably, introduction of dwarfing genes was an important outcome of the Green revolution, reducing lodging susceptibility by reducing plant height (Spielmeyer *et al*., 2002).

In conclusion, we observed that besides modifying growth, mechanical stimulation due to rubbing resulted in increased lignin deposition and also enhanced tensile strength leading to overall gain in the physical strength of the plant stem. Further, our results provide new insights on the lignin-flavonoid pathway after MIS and demonstrate the plasticity of the biochemical response. The ability to sense and respond to mechanical stimulation by thigmomorphogenesis needs to be explored, as it holds great potential for commercial application in horticulture for regulating plant growth without the use of chemical regulators (Börnke and Rocksch, 2018).

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Morphological and biochemical classification of Iranian mango germplasm collection by multivariate analysis: implications for breeding

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Abstract: This study was conducted at southern Iran with the aim to evaluate the phenotypic diversity of 84 mango cultivars by using morphological and biochemical traits. Two other industrial cultivars 'Longra' and 'Senderi' used as control of the study. Descriptive results indicated that the value of both quantitative and qualitative variables all the cultivars were lower than the 'Senderi'. The variability among cultivars was highly significant in the measured traits. The fruit weight, fruit length, stone weight, stone length, fruit beak, dry matter, and petiole length showed highly discriminating power. Pearson's correlation analysis revealed high variability due to the existence of significant positive and negative correlations among traits. Agglomerative hierarchical clustering confirmed remarkable variation in the studied germplasm and identified three major clusters with several sub-clusters. The 80.20, 70.11, and 100% of the quantitative variability was explained by principal component analysis (PCA), factor analysis (FA), and liner discriminant function (LDF) where fruit descriptors contributed most of the total variation, respectively. However, multivariate analysis proved that fruit related characters were most powerful to differentiate cultivars. The cultivars displayed distinct grouping by FA and LDF compared to PCA. The results revealed that the Iranian mango germplasm has a high potential for specific breeding project regarding fruit size and quality that should be further completed by a molecular marker analysis.

1. Introduction

The Mango (*Mangifera indica* L.), from *Anacardiaceae* family, is a fruit species native to Asia and grown comprehensively in tropical and subtropical countries, that originated as an allopolyploid from eastern India, Assam and Burma region (Krishna and Singh, 2007). Mangoes are extensively cultivated in the orchards or often planted in the fruit-gardens man-

ner in the southern region of Iran. The main areas of this region which is famous for the mango production are Hormozgan, Sistan and Balouchestan, and Kerman (Kahnuj and Jiroft) provinces.

The role of germplasm characterization in varietal development of crops as genotypes with desirable traits has been well identified and utilized in the crop improvement programs and also to determine evolutionary relationships (Piyasundara *et al*., 2008; Donkor *et al*., 2019). However, the accessibility of the germplasm depends mostly on the information available on characterization and evaluation. The investigation of plant material with desired traits by means of the morphological characterization by multivariate statistical techniques is an essential step for the effective utilization of crop germplasm (Piyasundara *et al*., 2008). Multivariate statistical techniques, PCA and cluster analysis are the popular multivariate techniques that widely applied to identify genetic diversity in germplasm of olives (Hagidimitiou *et al*., 2005), strawberry (Lavin *et al*., 2005), tea (Piyasundara *et al*., 2008), mangoes (Sennhenn *et al*., 2013; Jamil *et al*., 2015) and sour cherry (Ganopoulos *et al*., 2016). Among them, multivariate techniques guarantee the accurate interpretation of the information generated through characterization studies.

The local farmers believe that the mango cultivars cultivated at southern Iran may have been derived from Pakistan and Indian germplasms over 300-400 years ago. From the beginning of mango cultivation in the southern regions of Iran, mangoes have a local name by the farmers and the native people living based on their appearance and farmer's interest. Beside this, the area under mango cultivation is increasing each year in the southern regions of Iran, but the basic information regarding the analysis of genetic diversity and identification cultivars in order to improve breeding programs are lacking. Before any breeding programs on mango, there is the need to collect and characterize the local cultivars as plant material that are available in Iran. Moreover, the evaluation of morphological traits through phenotyping constitutes is a quick method to characterize the mango germplasm and being provide useful qualitative information for the breeding.

Therefore, the main objectives of the present study were (i) to identify the phenotypic diversity in 86 local mango cultivars of the Hormozgan province of southern Iran using morphological approaches based on multivariate statistical techniques, (ii) to evaluate specific traits for breeding and to progress future genetic resource conservation strategies.

2. Materials and Methods

Experimental areas survey

The experiments were conducted at Rudan, Siyahu, and Minab germplasm collections (RSM-collection), located in Hormozgan province of Iran (Fig. 1). Samplings were collected from RSM-collection during growing season for leaf (October to November), during flowering for flower (February and April) and fruit harvesting (July to September). Rudan, Siyahu, and Minab lie within latitude 57° 6' N, 27° 7' N and 49° 49' N and longitude 27° 7' E, 57° 11' E, and 34° 4' E and altitude 100, 300 and 700 m above sea level, respectively. The RSM-collection has an average annual rainfall of 227, 250 and 200 mm in two seasons (October and January) and main daily temperature of 28, 29 and 18°C, respectively (Metrological Service, Bandar Abbas, 2014-2017).

Plant material

A total of 84 cultivars from an inspection on mango gardens or scatter manner planted in RSM-collection followed by two industrial cultivars including 'Longra' and 'Senderi' as controls were comprised in the sampling procedure for characterization (Table S1). To assay adult trees of RSM-collection germplasm, three trees of each cultivar were randomly chosen and were labeled to use data collection. The labeled trees were at fruit-bearing capacity, healthy and in crop condition at beginning of the study. Sampling and morphological assessment of 49 variables as mango descriptors were programmed in the experimental collection for three consecutive years (2014-2017) at the same time of harvest season. Different horticultural practices, including fertil-

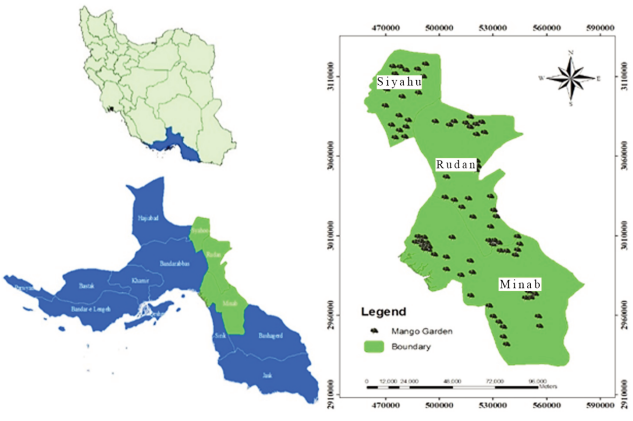

Fig. 1 - Geographical zones of the 84 mango cultivars followed by two industrial cultivars as controls used in this study from RSM- germplasm (Rudan, Siyahu, and Minab collection) located at southern of Iran (black trees indicate sampling locations).

izer application, spraying, irrigation and others, were performed at regular intervals each year.

Morpho-physiological analysis of traits

A total of 49 morphological and biochemical traits including 22 quantitative and 27 qualitative traits were scored following the guidelines system for mango descriptors published by International Plant Genetic Resources Institute (IPGRI), Rome for tree, leaf, flower and fruit traits (Mukherjee, 1989; PGRI, 2006). The quantitative traits were fruit length [FrLl], fruit breadth [FrBr], fruit skin thickness [FrSkTh], fruit weight [FrWe], flesh content (fruit flesh weight/fruit weight) [FrCo], fiber length [FrFi], stone length [StLe], stone weight [StWe], stone fiber length [StFiLe], pH [pH], TSS [TSS], titratable acidity [TiAc], total sugar [ToSu], moisture [MoSt], dry matter [DrMa] for fruit descriptors, leaf length [LeLe], leaf width [LeWi], leaf ratio [LeRa], petiole length [PeLe], petiole ratio [PeRa] for leaf descriptors, trunk circumference [TrCi] and canopy diameter [CaDi] for trunk descriptors. The qualitative traits analyzed were fruit shape [Frsh], flesh texture [FlCo], adherence [AdHe], fiber in pulp [FiPu], quantity of fiber [QuFi], stalk insertion [StIn], basal cavity [BaCa], beak [Be], beak type [BeTy], sinus [Si], sinus type [SiTy], groove stone [GrSt], shoulder [Sh], shoulder slope [ShSl], skin colour ripe fruit [SkCoRiFr] for fruit descriptors, inflorescence position [InPo], inflorescence shape [InSh], inflorescence colour [InCo], inflorescence hairiness [InHa], flower type [FlTy] for flower descriptors, leaf shape [LeSh], leaf colour [LeCo], leaf texture [LeTe], leaf tip [LeTi], leaf margin [LeMa] for leaf descriptors, trunk number [TrNu] and Tree habit [TrHa] for trunk descriptors.

For all three years, measurements performed by the same two persons to avoid errors due to individual variation. In addition, to diminish the environmental effects, all parameters were averaged over three years. Some measurements such as leaf, fruit and tree descriptors were performed in garden-head and others like flower, biochemical and physiological traits of fruit were measured at the laboratory of plant biotechnology of Hormozgan University. Total soluble solid and pH were determined using a digital refractometer (ATC1E-ATAGO, Japan), and pH meter (PL-500, Taiwan), respectively. Titratable acidity of freshly extracted juices and total sugar the fruits were calculated by using standard process procedure to AOAC (2000) and Omokolo *et al*. (1996), respectively. For morpho-metrical flower data collection, ten recently opened flowers from tree of each cultivar were randomly collected, pooled and conserved in ethanol (70%) until measurements. Afterward, morpho-metrical of flower was analyzed by using microscope (model: IX3).

Data analysis

Descriptive analysis (minimum, maximum, mean, standard deviation and coefficient of variation) for each of 49 studied traits were calculated and the test of normality was accomplished on data to approve ANOVA assumptions. Statistical differences in the Variations of each trait among the cultivars was computed by one-way analysis of variance (ANOVA) at $p \leq 0.01$ using SPSS 21.0 (SPSS, Inc., Chicago, IL, USA) after verifying normal distribution of dependent variables by Kolmogorov-Smirnov test. Within correlation analysis, the Pearson coefficient (parametric) was used to measure the correlation among quantitative traits. The principal component analysis (PCA), factor analysis (FA), liner discriminant function (LDF), and agglomerative hierarchical clustering (AHC) were directed to analyze data in order to visualize possible differences among the mango cultivars. In each case, a biplot was drawn based on most important components to facilitate the visualization of the results. For dendrogram construction, the combined data from both the quantitative and qualitative traits were considered. To estimate the genetic dissimilarity component, the Euclidean, and Ward's method was selected as chosen distance for the agglomerative hierarchical clustering. All multivariate analysis was performed using XLSTAT software (version 2016.2).

3. Results

Variance analysis of traits

The high morphological variation observed among studied cultivars. The mean squares of mango cultivars was significant for the LeLe, LeWi, LeCo, LeTe, FrLe, FrWe, FrSh, StLe, StWe, StFiLe, FiPu, FlFiLe, ToSu, DrMa, MoSt, Brix, InHa, CaDi, TrNu, and TrCi, but they were similar in the rest of the characteristics (data not shown). The variance variation in quantitative traits was more than qualitative traits.

Descriptive statistics

The result of descriptive analysis demonstrated the high variation of CV for SiTy (95%), Si (94%), StFiLe (67%), pH (64%), BeTy (62%), TrNu (62%), LeCo (55%), InHa (52%), FrSh (52%), TrCi (42%), QuFi (41%), TrHa (41%), LeTu (40%) Be (36%), FlTy (35%),

FlFiLe (34%), StIn (34%), InSh (33%) and InCo (31%), respectively. In contrast, the other variables revealed low coefficient of variation (<30%) (Supplementary Table S2). In the traits mentioned above, the high they produced the CV, the greatest was the variability regarding the fruit descriptor was proportional to the CV. For leaf descriptor, the most relevant traits were: LeCo, and LeTu whereas InHa, InCo, InSh, and FlTy were the most relevant character for the flower descriptor. The studied cultivars exhibited high morphological diversity, with some quantitative and qualitative traits.

Correlations for quantitative variables

Pearson's correlation (parametric) among 22 morphological of quantitative characters were presented in Table 1. Results demonstrated that the 28 positives and 11 negative significant correlations. A significant positive correlation was obtained between FiLe and StLe (r= 1.00), and FrBr and FrWe (r= 0.734). In contrast, highest negative correlation was resulted among MoSt and DrMa (r= -0.996). Regarding fruit descriptors, the results of the paired linear correlation indicated that FrWe was positively correlated with FrBr, FrLe, StWe, and TSS while was negatively correlated with CaDi.

In the present study, significant correlation was found between fruit weight and other quality variables specially TSS. Additionally, the Pearson correlation of FrBr with FrWe, FlCo, and StWe was positive significant whereas was negative significant with TSS (Table 1). Furthermore, significant positive correlations was also observed among: FlCo and FrSkTh (r= 0.244), TSS and FrSkTh (r= 0.347), FlCo and FrWe (r= 0.461), FrWe and StLe (r= 0.247), FrWe and StWe (r= 0.558), StLe and TSS (r= 0.277), StWe and MoSt (r= 0.229), TiAc and ToSu (r= 0.294), and ToSu and DrMa (r= 0.294) while significant negative correlation was detected between FlCo and StWe (r= -0.431).

Principal component analysis of quantitative variables

According to apply Kaiser's criterion ("Eigenvalue" >1) (Kaiser, 1958), PCA analysis of the 22 quantitative traits resulted in 9 components for explaining total of variation among cultivars. PCA revealed the first nine

Table 1 - Correlation coefficients (Pearson) among 22 quantitative traits in 86 mango cultivars

	FrLe	FrBr	FrSkTh	FrWe	FlCo	FiLe	StLe	StWe	StFiLe	pH	TSS	TiAc	ToSu	MoSt	DrMa	LeLe	LeWi	LeRa	PeLe	PeRa	TrCi	CaDi
FrLe	1																					
FrBr	**0.426**	1																				
FrSkTh	0.44	-0.148	1																			
FrWe	**0.456**	**0.734**	0.022	1																		
FlCo	**0.243**	**0.372**	**0.244**	**0.46**	1																	
FiLe	-0.088	-0.087	-0.054	0.015	0.035	1																
StLe	**0.591**	0.094	-0.025	**0.247**	0.1	1	1															
StWe	**0.239**	**0.381**	-0.189	**0.558**	**-0.434**	0.035	0.187	1														
StFiLe	0.63	0.011	0.115	0.112	0.056	0.103	0.087	0.027	1													
pH	-0.213	-0.113	-0.105	-0.107	0.023	-0.364	-0.188	-0.115	-0.064	1												
TSS	**0.304**	**-0.32**	**0.347**	-0.18	0.029	-0.029	**0.277**	-0.155	-0.044	-0.062	1											
TiAc	0.114	-0.015	-0.123	-0.039	0.026	0.055	0.112	-0.074	0.106	**-0.383**	0.028	1										
ToSu	-0.65	**-0.23**	0.08	-0.138	0.105	-0.071	-0.002	-0.191	0.005	0.098	**0.471**	**0.294**	1									
MoSt	0.091	0.116	0.113	0.165	-0.094	-0.099	0.013	**0.229**	0.041	-0.193	-0.189	-0.155	**-0.29**	1								
DrMa	-0.073	-0.105	-0.11	-0.16	0.082	0.099	0.005	-0.211	-0.044	0.201	0.195	0.149	0.294	-0.996	1							
LeLe	0.205	0.142	0.108	0.069	0.099	-0.219	0.018	-0.033	0.047	**-0.236**	0.138	0.089	-0.03	-0.013	0.016	1						
LeWi	0.101	0.14	0.023	0.124	0.106	-0.361	-0.092	0.005	-0.067	-0.209	-0.041	0.079	-0.024	-0.023	0.039	**0.681**	1					
LeRa	0.113	0.048	0.113	-0.019	0.019	0.221	0.125	-0.047	0.132	-0.039	**0.215**	0.004	0.001	0.018	-0.033	**0.429**	**-0.342**	1				
PeLe	0.103	0.159	0.072	0.048	0.108	-0.12	-0.025	0.008	0.058	-0.198	0.03	0.093	-0.018	0.026	-0.022	**0.651**	**0.388**	**0.347**	1			
PeRa	-0.032	0.076	0.009	0.018	0.084	0.026	-0.017	0.033	0.013	-0.048	-0.094	0.031	-0.021	0.047	-0.044	-0.034	-0.092	0.055	**0.722**	1		
TrCi	-0.055	0.004	-0.084	0.118	0.046	-0.262	-0.138	0.128	-0.225	0.078	0.029	**-0.275**	-0.075	-0.011	0.018	-0.148	0.077	-0.056	-0.069	0.057	1	
CaDi	**-0.22**	-0.048	-0.085	0.067	-0.099	0.121	-0.152	0.118	-0.012	0.031	-0.54	-0.113	0.011	-0.095	0.102	-0.09	-0.132	0.105	-0.041	0.014	**0.645**	1

For full names of traits see Morpho-physiological analysis of traits head in Materials and Method section.

(PCs) with eigen values greater than value 1, which could explain 80.20% of the total variation (Table 2). PC1, which accounted for 15.91% of the total variation, was strongly associated with fruit traits, such as fruit length, fruit weight, fiber length, stone length, and fruit breadth. Hence, the cultivars with high value of PC1 have lower biochemical traits of fruit as well as smaller tree size (canopy diameter). PC2 accounting for 13.33% of the total variation was positively correlated with total solid soluble, fruit dry matter, trunk circumference and leaf traits, while negatively correlated with the most of fruit parameters. PC3 had high contributing factor loading from petiole length, leaf length, leaf width, petiole ratio, trunk circumference and contributed 11.37% of the total variation. PC3 suggested that leaf traits could be located in one index. PC4, accounted for 8.79% of the total variation, was most determined by the traits of fruit dry matter, fruit breadth, fruit weight, flesh content and pH. PC5 up to PC9 explained 7.28%, 7.08%, 5.93%, 5.51%, and 5.21% of the total variation, respectively. However; PC5 represents mainly flesh content, fruit skin thickness, fruit weight, leaf ratio, and petiole ratio; PC6 explains canopy diameter, TSS, pH, petiole length, and petiole ratio; PC7 describes leaf length, pH, and stone weight; PC8 illustrates stone fiber length, leaf ratio, trunk circumference, and titratable acidity; PC9 most demonstrates total sugar, canopy diameter, TSS, and stone weight.

In addition, PCA-biplot based on PC1 and PC2 exhibited that the cultivars had wider variation for quantitative traits and thus the biplot did not produce a distinct grouping among cultivars (Fig. 2A). According PCA biplot, negative values for PC1 indicate cultivars with high content of dry matter and total sugar as well as lower pH and smaller canopy diameter; however, the cultivars which were placed in the green line rectangle belong this group (Fig. 2A).

Table 2 - First 9 components from the PCA of 22 quantitative traits in 86 mango cultivars

Quantitative traits	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
FrLe	0.77	0.08	-0.32	0.04	0.07	-0.06	0.08	-0.08	-0.03
FrBr	0.61	-0.32	0.14	0.51	0.1	0.13	-0.02	0.14	-0.02
FrSkTh	0.07	0.23	0.06	-0.33	0.63	0.00	0.11	0.00	0.28
FrWe	0.68	-0.37	-0.05	0.44	0.20	0.12	0.05	0.13	0.22
FlCo	0.31	0.21	0.01	0.39	0.71	0.12	-0.27	0.08	-0.07
FiLe	0.64	0.15	-0.61	-0.16	-0.16	0.02	-0.03	-0.15	-0.18
StLe	0.64	0.15	-0.61	-0.16	-0.16	0.02	-0.03	-0.15	-0.18
StWe	0.40	-0.51	-0.06	0.08	-0.46	0.06	0.27	-0.04	0.30
StFiLe	0.17	0.07	0.01	-0.13	0.03	0.17	-0.2	0.54	0.06
pH	-0.43	-0.10	-0.16	0.22	0.23	0.33	0.21	-0.17	-0.33
TSS	0.08	0.58	-0.38	-0.30	0.18	-0.03	0.29	-0.20	0.30
TiAc	0.15	0.37	-0.03	0.06	-0.36	-0.26	-0.53	0.30	0.25
ToSu	-0.19	0.51	-0.26	0.07	0.09	-0.03	-0.11	-0.04	0.50
MoSt	0.30	-0.58	0.27	-0.6	0.18	-0.13	-0.08	-0.01	0.07
DrMa	-0.29	0.58	-0.28	0.61	-0.19	0.13	0.09	-0.01	-0.06
LeLe	0.43	0.47	0.56	0.01	-0.05	-0.18	0.42	0.06	-0.09
LeWi	0.25	0.21	0.49	0.31	0.00	-0.62	0.21	-0.18	0.03
LeRa	0.24	0.33	0.12	-0.34	-0.05	0.56	0.31	0.34	-0.12
PeLe	0.39	0.39	0.68	-0.02	-0.15	0.3	-0.05	-0.32	0.06
PeRa	0.15	0.09	0.37	-0.03	-0.13	0.55	-0.45	-0.52	0.13
TrCi	0.15	0.51	0.29	-0.16	-0.15	0.02	-0.09	0.32	-0.31
CaDi	-0.21	-0.14	0.01	0.14	-0.18	0.37	0.34	0.21	0.44
Eigenvalue	3.50	2.89	2.50	1.93	1.60	1.56	1.30	1.21	1.15
Variability (%)	15.91	13.13	11.37	8.79	7.28	7.08	5.93	5.51	5.21
Cumulative	15.91	29.04	40.41	49.20	56.47	63.65	69.48	74.99	80.20

For full names of traits see Morpho-physiological analysis of traits head in material and method section.

The highest positive values for PC1 displayed cultivars with high fruit weight, fruit length, fruit breadth, stone weight and high of fruit moisture as well as lower total sugar and dry matter, which were positioned in the green line oval, as shown in figure 2A. These cultivars can be applied as parents in the mango breeding program as a source of genes for a greater fruit size in a second cycle of recurrent selection.

Principal component analysis of qualitative variables

According to PCA, the cultivars were quantitatively distinct based on qualitative variables. Considering the Kaiser's criterion ("Eigenvalue" >1), nine significant components were obtained that explained 64.26% of the total variation (Table 3). PC1 was associated with tree habit, basal cavity, inflorescence shape, inflorescence colour and leaf shape accounted for 12.70% of the total variation. PC2 had high contributing factor loading from fruit traits such as stalk insertion, beak, beak type, shoulder slope and contributed 10.43% of the total variation. The more PC3 value was positively correlated to sinus and sinus

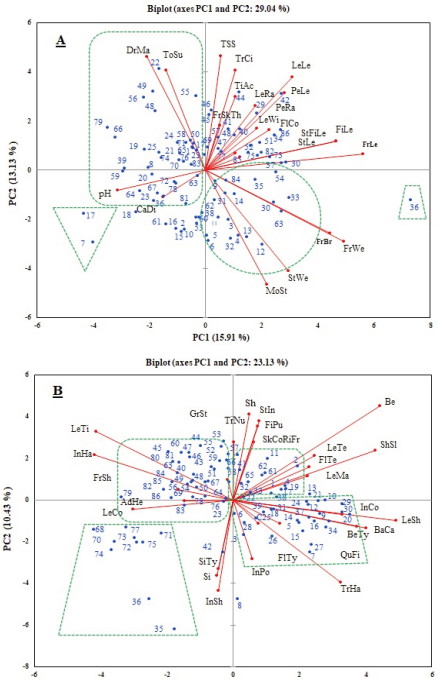

Fig. 2 - A) PCA biplot of the 22 quantitative traits with regard to the first two principal components. B) PCA biplot of the 27 qualitative traits with regard to the first two principal components among 86 mango cultivars.

Table 3 - First 9 components from the PCA of 22 quantitative traits in 86 mango cultivars

Quantitative traits	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
FrSh	-0.13	-0.05	0.05	-0.19	0.04	-0.09	-0.02	-0.56	-0.14
FtTe	-0.09	0.17	0.02	0.00	0.20	-0.04	-0.79	-0.10	-0.08
AdHe	0.05	0.06	-0.25	-0.20	-0.18	0.47	0.11	-0.37	0.04
FiPu	-0.06	0.13	-0.07	0.70	0.12	0.01	0.10	0.33	-0.02
QuFi	0.28	0.06	0.01	0.00	-0.01	0.05	-0.17	0.07	-0.80
StIn	-0.10	0.80	0.01	0.08	-0.29	0.13	0.06	0.01	-0.08
BaCa	0,60	-0.04	-0.19	0.05	0.08	0.09	-0.04	0.11	0.09
Be	0.12	0.66	-0.23	0.06	0.22	-0.06	-0.23	0.05	0.22
BeTy	0.25	0.40	0.23	-0.48	0.11	-0.11	-0.06	0.13	0.30
Si	-0.04	-0.06	0.95	-0.09	0.05	0.04	-0.02	-0.01	0.00
SiTy	-0.07	-0.02	0.95	-0.09	0.04	0.03	-0.06	0.00	0.03
GrSt	-0.22	-0.14	0.03	-0.04	0.59	0.12	-0.20	-0.04	-0.10
Sh	0.01	-0.02	-0.11	0.79	0.05	-0.09	-0.12	-0.24	0.05
ShSl	0.21	0.44	0.17	0.28	0.09	0.01	-0.49	-0.07	0.18
SkCoRiFr	0.01	0.29	0.11	0.17	0.27	-0.22	0.34	-0.07	-0.42
InPo	0.03	-0.04	0.04	-0.23	-0.06	-0.07	0.05	0.70	-0.17
InSh	0.26	-0.65	0.17	0.17	-0.08	0.14	0.03	0.07	0.39
InCo	0.51	0.11	-0.01	-0.22	0.15	-0.18	0.16	-0.31	0.02
InHa	-0.73	-0.13	-0.21	-0.14	0.09	-0.07	-0.13	-0.05	0.12
FlTy	0.24	0.13	0.13	0.10	0.01	0.89	-0.10	-0.03	0.02
LeSh	0.47	0.11	-0.21	-0.19	0.25	-0.02	-0.22	0.28	0.10
LeCo	-0.33	-0.09	-0.05	-0.16	0.25	0.68	0.17	0.17	-0.09
LeTe	0.00	0.04	0.01	0.05	0.72	-0.13	0.00	0.11	0.13
LeTi	-0.74	0.06	0.07	0.10	0.14	-0.02	0.11	-0.02	0.09
LeMa	0.23	-0.04	0.07	0.11	0.67	0.08	0.12	-0.17	-0.06
TrNu	-0.03	0.25	-0.12	0.01	0.25	0.03	0.52	-0.10	0.17
TrHa	0.65	-0.33	-0.02	-0.08	0.07	-0.07	-0.01	0.08	-0.08
Eigenvalue	3.43	2.82	2.44	1.77	1.59	1.53	1.37	1.27	1.14
Variability (%)	12.70	10.43	9.05	6.54	5.89	5.68	5.06	4.70	4.21
Cumulative	12.70	23.13	32.17	38.71	44.61	50.28	55.35	60.04	64.26

For full names of traits see Morpho-physiological analysis of traits head in material and method section.

type belong to fruit descriptor that explained the 9.05% of the total variation. PC4 explained 6.54% of the total variation with highest positive correlations to fiber in pulp and shoulder. PC5 up to PC9 explained 5.89%, 5.68%, 5.06%, 4.70%, and 4.21% of the total variation, respectively. However; PC5 represents mainly leaf texture, leaf margin and groove stone; PC6 explains flower type, leaf colour and adherence; PC7 describes tree number and skin colour ripe fruit; PC8 illustrates inflorescence position and fiber in pulp; PC9 most demonstrates beak type, inflorescence shape and quantity of fiber (Table S5). Figure 2B represents PC1 and PC2 plotted on a bi dimensional plane. In contrast to quantitative traits, qualitative traits indicated clear cut differences between individuals and forms a spectrum of phenotypes, which means that they are highly heritable and the environment has very little influence on the phenotype of these traits. Hence, PCA-biplot exhibited that the cultivars scattering in all the quarters, and the association between traits and cultivars for qualitative traits was more discriminator than quantitative traits. The negative values for PC1 indicate cultivars with high content of leaf tip, inflorescence hairiness, fruit shape, and groove stone as well as lower leaf colour, inflorescence shape, fruit adherence, and fruit sinus. Cultivars were positioned in the green line rectangle belong this group. These could be target characteristics depending on different purposes in breeding. The highest positive values for PC1 illustrate cultivars with high tree habit growth, inflorescence position and colour, quantity fiber of fruit, leaf shape, and basal cavity of fruit, which were positioned in the green line parallelogram. In contrast, the highest positive values for PC2 show cultivars with high fruit traits, as shown in figure 2B.

Dendrogram using agglomerative hierarchical clustering

The dendrogram showed three main groups; the C1 is included of 31 cultivars, the C3 contained of 54 cultivars, and finally the C2 comprised of 4 cultivars (Fig. 3). Clustering analysis resulted in the high level of morphological and biochemical variation among cultivars, as confirmed by descriptive and variance analysis. It was considerable that there was no relationship between clustering pattern and geographical distribution. One of its reason can be related to synonyms, homonyms and misnames. Indeed at 89.66% dissimilarity cultivars, which were placed in C1, were separated from others by their small fiber length (centroid= 5.15), lowest TSS (centroid=10.64), lowest

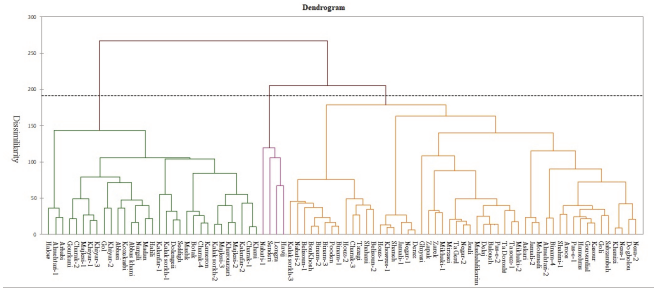

Fig. 3 - Dendrogram using AHC for 86 mango cultivars based on 22 quantitative and 27 qualitative traits.

leaf ratio (centroid= 4.23), their absence groove stone (centroid= 0) and also by their highest stone weight (centroid= 32.44). At 67.66% dissimilarity the other cultivars were divided into two groups: the cultivars, which were located in C2, characterized by their highest fruit dry matter (centroid= 17.93), larger trunk circumference (centroid= 1.88), presenting groove stone in fruit (centroid= 0.020), and having poor hairiness in flower inflorescence (centroid= 2.33), and the cultivars (C3) having highest fruit length (centroid= 9.39), fruit weight (centroid= 162.69), and also having highest value in the most of traits.

Factor analysis

The all dataset of both quantitative and qualitative variables was subjected to KMO (Kaiser-Meyer-Olkin) and Bartlett's test of sphericity. KMO (0.37) less than 0.5 and no significant Bartlett's test of sphericity showed that the all data were not very suitable for FA. KMO was found to be 0.58 for all dataset and the Bartlett's test of sphericity was significant (Chi-square = 342.459; df = 171, $p < 0.05$), when the dataset of two variables was separately used for FA. Based on FA, 22 quantitative traits were divided in eight factors (Fs) that had eigenvalue more than one and explained 70.17% of variance (Table 4). Percent of variance of each factor is showing importance of that factor. Factor loading more than 0.50 was considered as significant factor loading for each factor. F1 was included for fruit length, fruit breadth, fiber length, and stone length and it could explain 15.11% of total variation in the dependent structure for, and its suggested name is fruit yield. F2 accounted for 12.45% of total variability and was consisted of high total soluble solid, fruit dry matter as well as low fruit moisture which was named fruit taste. F3 justified 11.06% of total variability which is strongly influ-

enced by higher stone length and fiber length as well as lower petiole and leaf length, and it was suggested name fruit and weak leaf morphology. F4 accounted for 8.30% of variability and was mainly explained by moisture and dry matter which was named fruit dry matter. F5 was named leaf morphology and had high leaf width as well as low petiole ratio which contributed to 6.62% of total variation. F6 was introduced as fruit flesh and had high fruit flesh content contributed 6.44% of total variation. F7 and F8 had high contributing loading from titratable acidity which was named fruit titratable acidity.

It was noticed that the grate variation based on eight factors was observed among cultivars, but it could not produce very suitable or distinct grouping due to generate overlapping or same factors such as titratable acidity and leaf morphology in FA. This aspect has obviously been shown by FA-biplot based on first Fs extracted from quantitative variables in figure 4A. FA-biplot indicated that cultivars scattered in all the quarters and thus cannot generated proper grouping. To overcome this, quartimax rotation is a statistical technique used at one level of factor analysis as an attempt to clarify the relationship among factors. In Table 4, it can be seen that the quartimax as an oblique rotation displayed a higher efficacy to create clear patterns of results in FA where the factors are indeed correlated. However, in factor analysis by means of quartimax rotation and on the base of factor loading larger than 0.5, five rotation factors (RF) were identified and they all together justify 53.46% of existent variations among the traits. RF1 up to RF5 were called fruit size factor, dry matter factor, leaf size factor, fruit weight factor, petiole size and also were accounted for 12.62, 11.11, 10.11, 10.68, and 8.92% respectively. Result indicated that quartimax rotation minimized the complexity of the factor loadings to make the structure simpler to interpret, as shown in figure 4B. According to RFA-biplot cultivars were quantitatively separated to six distinct groups in comparison to FA-biplot. The cultivars 22 (‘Almehtari-2’) and 48 (‘Binam-4’) were separated into distinct group near the positive ends of the RF2 axis, and were correlated with respect to higher

Table 4 - Correlation coefficients explained by the first eight factor analysis (Fs) and quartimax rotation for 22 quantitative traits in 86 mango cultivars

Quantitative variables	Factors without rotation								Factors after Quartimax rotation				
	F1	F2	F3	F4	F5	F6	F7	F8	RF1	RF2	RF3	RF4	RF5
FrLe	**0.699**	0.074	0.258	0.039	0.057	0.059	0.028	0.039	0.639	-0.047	0.203	0.326	0.048
FrBr	**0.563**	-0.276	-0.14	0.44	-0.149	0.018	0.084	-0.15	0.099	-0.119	0.092	**0.771**	0.055
FrSkTh	0.059	0.153	-0.046	-0.178	-0.007	0.382	0.013	0.088	0.079	-0.01	0.100	-0.141	0.158
FrWe	**0.695**	-0.357	0.03	0.485	-0.148	0.100	0.122	-0.087	0.275	-0.143	0.022	**0.880**	-0.026
FlCo	0.328	0.211	-0.03	0.443	-0.276	**0.705**	-0.224	-0.084	0.102	0.348	0.080	0.495	0.205
FiLe	**0.669**	0.150	**0.615**	-0.17	0.019	-0.121	-0.088	0.144	**0.928**	0.005	0.007	0.094	0.023
StLe	**0.669**	0.150	**0.615**	-0.17	0.019	-0.121	-0.088	0.144	**0.928**	0.005	0.007	0.094	0.023
StWe	0.404	-0.492	0.062	0.065	0.042	-0.518	0.269	0.061	0.200	-0.417	-0.066	0.415	-0.144
StFiLe	0.128	0.035	-0.001	-0.06	-0.058	0.014	-0.027	-0.151	0.100	-0.029	0.024	0.028	0.110
pH	-0.347	-0.064	0.11	0.133	-0.216	0.100	0.189	0.139	-0.207	0.182	-0.346	-0.028	-0.056
TSS	0.087	**0.574**	0.362	-0.33	0.100	0.234	0.156	0.267	**0.500**	0.296	0.146	-0.467	0.123
TiAc	0.140	0.366	0.035	0.50	0.219	-0.311	**-0.561**	**-0.567**	0.182	0.226	0.339	-0.079	-0.024
ToSu	-0.155	0.405	0.18	0.025	0.009	0.060	-0.103	-0.006	0.092	0.394	0.035	-0.235	0.010
MoSt	0.302	**-0.635**	-0.251	**-0.539**	0.150	0.250	-0.162	0.023	0.051	**-0.921**	0.028	-0.006	0.055
DrMa	-0.290	**0.638**	0.262	**0.552**	-0.143	-0.267	0.170	0.049	-0.038	**0.929**	-0.023	0.018	-0.065
LeLe	0.438	0.473	**-0.569**	-0.045	0.312	0.011	0.338	-0.044	0.008	0.001	**0.863**	0.035	0.312
LeWi	0.252	0.215	-0.500	0.331	**0.631**	0.010	-0.004	0.286	-0.187	0.024	**0.867**	0.203	-0.220
LeRa	0.260	0.327	-0.119	-0.447	-0.409	0.023	0.479	-0.447	0.234	-0.028	0.018	-0.174	**0.687**
PeLe	0.407	0.397	**-0.702**	-0.122	-0.270	-0.205	-0.066	0.221	-0.133	-0.019	**0.509**	0.158	**0.774**
PeRa	0.160	0.088	-0.389	-0.107	**-0.633**	-0.257	-0.410	0.362	-0.159	-0.025	-0.112	0.195	**0.723**
TrCi	0.119	0.381	-0.204	-0.138	0.026	-0.042	-0.019	-0.199	0.042	0.114	0.327	-0.147	0.282
CaDi	-0.156	-0.083	-0.009	0.057	-0.142	-0.108	0.187	-0.050	-0.145	0.033	-0.179	0.025	0.013
Eigenvalue	3.325	2.741	2.434	1.826	1.457	1.417	1.199	1.040	>1	>1	>1	>1	>1
Variability (%)	15.112	12.459	11.063	8.301	6.62	6.411	5.449	4.728	12.624	11.119	10.117	10.686	8.923
Cumulative	15.112	27.571	38.633	46.934	53.554	59.996	65.445	70.173	12.624	23.743	33.86	44.546	53.469

For full names of traits see Morpho-physiological analysis of traits head in material and method section.

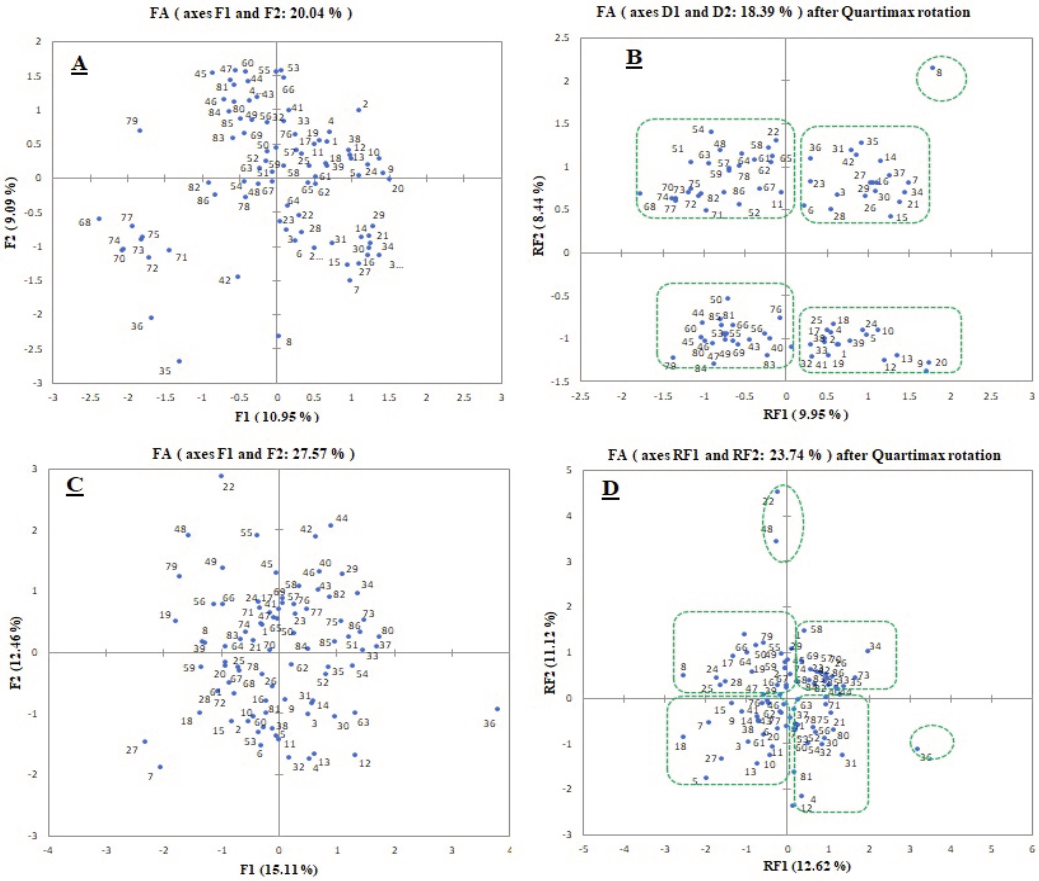

Fig. 4 - Factor analysis-biplot of the 22 quantitative (A) and 27 qualitative traits (C) with regard to the first two factors and with regard to the first two factors after quartimax rotation (RF) (B, D) among 86 mango cultivars, respectively.

fruit dry matter as well as lower fruit moisture which positioned in green line oval. Selection of these cultivars is a further advantage, as they have important postharvest characters for mango fruit breeding to extend their shelf-life. In contract, the 'Senderi' as control in green line circle was clearly diverged from the other cultivars that were located at the positive ends of RF1 axis, and was highly correlated with respect to fruit length, flesh content and stone length and the 'Kalanfar-2' was the most closet cultivar to control, as shown in figure 4B. Therefore, crosses between 'Kalanfar-2' and other cultivars with which 'Kalanfar-2' produced fertile F1 progeny would potentially allow the production of segregating populations for QTL analysis.

Additionally, FA was applied to investigate variation in qualitative variables. Based on FA, 27 qualitative traits were divided in six factors that had eigenvalue more than one and explained 41.39% of total variation (Table 5). Primary result of FA clearly proved that variation in quantitative variables is more complexity than qualitative variable among cultivars and thus reduced the number of dimensions and discovered simple patterns in the pattern of relationships among the variables in comparison to PCA. Also, FA confirmed that the variation in qualitative variables less than quantitative variables among cultivars while PCA wasn't able to detect it. The six factors of qualitative variables namely, (F1) fruit and leaf morphology, (F2 and 3) fruit sinus, (F4) flower type, (F5) fruit stalk insertion, and fruit flesh texture were accounted for 10.95, 9.08, 7.98, 5.13, 4.47, and 3.755% of total variation, respectively. Like FA for quantitative variables, same factors observed to separate cultivars base on the qualitative variables and thus it could not generate distinct grouping, as shown in figure 4C. FA-biplot indicated that cultivars scattered in all the quarters and thus cannot generated proper grouping. Hence, to clarify the relationship among factors, RFA was used. Base on the factor loading larger than 0.5, five RFs were identified and they all together justify 37.59% of existent variations (Table 5). RF1, called as fruit, flower, and growth morphology factor, which accounted for 9.94% of the

Table 5 - Correlation coefficients explained by the first six factor analysis (Fs) and quartimax rotation for 27 qualitative traits in 86 mango cultivars

Qualitative variables	Factors without rotation						Factors after Quartimax rotation				
	F1	F2	F3	F4	F5	F6	RF1	RF2	RF3	RF4	RF5
FrsH	-0.186	0.023	0.150	-0.112	0.138	-0.051	-0.182	0.120	-0.119	-0.088	0.149
FtTe	0.410	0.024	0.469	0.269	-0.075	**0.693**	0.088	0.224	**0.628**	0.111	0.190
AdHe	-0.175	0.136	-0.315	0.092	0.347	-0.017	-0.068	-0.195	-0.372	0.268	0.113
FiPu	0.136	0.353	0.115	0.245	-0.370	-0.155	-0.178	-0.225	**0.501**	0.049	-0.063
QuFi	0.148	-0.146	0.017	-0.070	0.076	-0.035	0.203	0.108	-0.013	-0.027	0.045
StIn	0.159	0.369	0.170	-0.008	**0.504**	-0.018	-0.040	-0.078	0.027	0.135	**0.646**
BaCa	0.482	-0.105	-0.339	0.051	-0.088	-0.080	**0.551**	-0.208	0.100	0.111	-0.137
Be	**0.734**	0.453	0.185	0.052	0.199	0.033	0.348	-0.253	**0.529**	0.099	0.625
BeTy	0.491	-0.272	0.101	-0.191	0.328	0.018	**0.564**	0.250	0.014	-0.042	0.315
Si	-0.141	**-0.780**	**0.603**	0.115	0.085	-0.146	-0.013	**0.966**	0.072	-0.005	-0.153
SiTy	-0.126	**-0.725**	**0.618**	0.122	0.096	-0.101	-0.031	**0.938**	0.100	0.001	-0.110
GrSt	-0.008	0.009	0.232	0.116	-0.144	-0.092	-0.137	0.116	0.245	-0.007	-0.034
Sh	0.122	0.429	0.154	0.269	-0.433	-0.110	-0.250	-0.294	**0.575**	0.036	-0.063
ShSl	**0.623**	0.085	0.306	0.247	-0.002	0.126	0.311	0.068	**0.620**	0.164	0.268
SkCoRiFr	0.101	0.174	0.306	-0.194	0.029	-0.374	-0.064	0.048	0.181	-0.223	0.310
InPo	0.037	-0.266	-0.121	-0.085	-0.030	0.114	0.208	0.098	-0.133	-0.050	-0.173
InSh	-0.129	-0.434	-0.382	0.279	-0.475	0.018	0.117	0.009	-0.053	0.514	**-0.815**
InCo	0.392	-0.119	-0.070	-0.257	0.111	-0.220	0.468	0.000	-0.015	-0.144	0.160
InHa	**-0.507**	0.278	0.188	0.015	-0.043	0.181	**-0.619**	-0.028	-0.065	-0.071	0.048
FlTy	0.114	-0.111	-0.298	0.853	0.443	-0.209	0.124	0.021	-0.025	**0.962**	-0.057
LeSh	**0.633**	-0.130	-0.207	-0.001	-0.037	0.016	**0.654**	-0.119	0.203	0.062	-0.007
LeCo	-0.396	0.045	-0.130	0.349	0.185	-0.149	-0.351	-0.006	-0.228	0.377	-0.097
LeTe	0.339	0.078	0.292	0.014	-0.272	-0.310	0.103	0.031	**0.502**	-0.141	0.054
LeTi	-0.515	0.345	0.389	0.082	-0.027	-0.095	**-0.743**	0.058	0.079	-0.056	0.155
LeMa	0.293	0.007	0.163	0.060	-0.174	-0.377	0.146	0.029	0.357	-0.038	0.010
TrNu	0.030	0.269	0.022	-0.091	0.089	-0.270	-0.073	-0.168	0.018	-0.053	0.238
TrHa	0.361	-0.400	-0.322	-0.082	-0.185	-0.049	**0.592**	-0.001	-0.033	-0.042	-0.345
Eigenvalue	2.957	2.453	2.156	1.387	1.209	1.014	>1	>1	>1	>1	>1
Variability (%)	10.952	9.086	7.985	5.139	4.474	3.755	9.947	8.443	7.57	5.1	6.478
Cumulative	10.952	20.038	28.023	33.161	37.64	41.394	9.947	18.39	25.961	31.12	37.59

For full names of traits see Morpho-physiological analysis of traits head in Materials and Methods section.

total variation. RF2 with the 8.44% of the variance reflected the fruit sinus dimension and therefore, being classified as fruit sinus. RF3 with the 7.57% of the total variance showed the high flesh texture, fiber in pulp, beak, shoulder, and leaf texture dimension and therefore, being classified as fruit morphology. RF4 with 5.16% of the variance classified as flower type factor due to high factor loading for flower type. Finally, RF5 demonstrated the stalk insertion and inflorescence shape factor which accounted for 6.47% of the variance. However, the quartimax rotation used in the factor analysis had excellent discrimination among cultivars based on factors criterion compared to FA without quartimax rotation. This aspect was confirmed by RFA-biplot where showed the cultivars separated into five distinct groups and thus offered the excellent grouping comparison with FA-biplot (Fig. 4D). The 'Nabati-1' (8) cultivar was qualitatively separated into single group near the positive ends of the RF2 and RF1axes, which positioned in green line circle, and was correlated with respect to higher fruit, flower, and growth morphology and fruit sinus classified-factors. So, the 'Nabati-1' cultivar can be considered as favorable genetic material for mango breeding via effective phenotypic selection of its correlated-traits (traits obtained by RF1 and RF2) and high expected genetic gain from selection for its correlated-traits can be achieved, as confirmed by cluster analysis.

Discriminant function analysis

The LDF from both quantitative and qualitative variables to classify the cultivars formed on RSM-collection population (Rudan, Siyahu and Minab) was

analyzed separately. Walk's lambda test was found to be 0.21 and 0.04 (p-value < 0.0001, alpha 0.05) for quantitative variables and qualitative variables, respectively. However, Walk's lambda test was significant for two discriminant function obtained from quantitative and qualitative variables, and thus there was low correlation between independent (measured variables) and dependent (population) variable to compute new directions (canonical variates or discriminant functions) in which the groups are best separated by LDF.

The LDF across the 22 quantitative variables originated from RSM-collection is shown in Supplementary Table 3. The first two discriminant functions were able to capture 100% of the total variance. Function 1 explains 82.37% of the total variance and function 17.62% of the total variance. Major contributors to discriminate among different cultivars in function 1 are the fruit length, fruit breadth, fruit weight, fiber length, stone length, stone weight, stone fiber length, and canopy diameter; meanwhile the leaf length, leaf ratio, petiole length, fruit weight, stone weight, TSS, fruit total sugar, fruit dry matter, and trunk circumference are major contributors in function 2. Therefore, these functions might represent the relationship of cultivars in each class (Rudan, Siyahu and Minab) with high efficiency at RSM-collection (Fig. 5A). Considering the 1st and 2nd discriminant functions, the cultivars presented at all three classes had closer genetic relationships; hence, there was a large overlap of the cultivars belonging to Minab and Rudan due to the stronger relationship between the classes. However, the results of confusion matrix for the cross-validation and Mahalanobis distance based on quantitative traits showed that the 55.81% (48 number) of total cultivars belonged to their original classes and the rest cultivars (44.19% = 38 number) were distributed among the three classes (Table S3).

Additionally, LDF across the 27 qualitative variables was performed (Table S3). The first two discriminant functions were able to capture 100% of the total variance. Function 1 explains 63.48% of the total variance and function 36.51% of the total variance. Function 1 explains 82.37% of the total variance and function 17.62% of the total variance. Major contributors to discriminate among different cultivars in function 1 are the leaf shape, tree habit. basal cavity, beak type, inflorescence hairiness, inflorescence colour, shoulder slope, and quantity of fiber; meanwhile the inflorescence shape, skin colour ripe fruit, beak fruit, stalk insertion, and flesh texture are major contributors in function 2. The cultivars presented at all three classes were qualitatively grouped well, with slight overlapping of between classes in compared grouping based on quantitative variables (Fig. 5B). However, the few cultivars distributed in among classes due to the slighter relationship

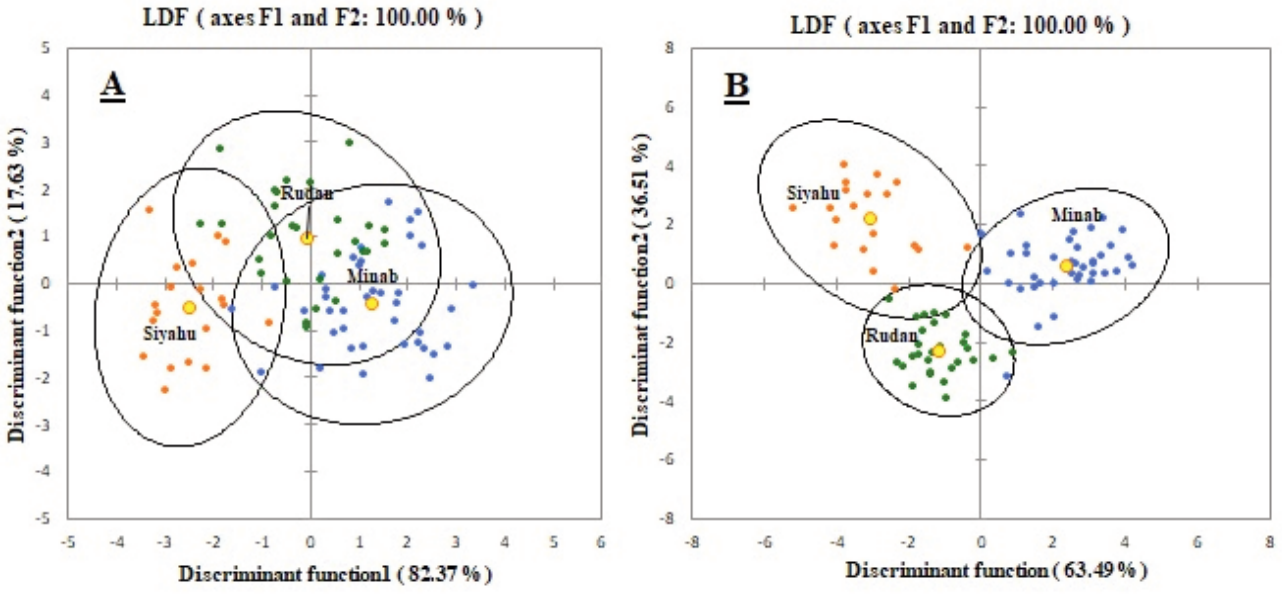

Fig. 5 - Liner discriminant functions-biplot of the 22 quantitative traits (A) and 27 qualitative traits (B) with regard to the two discriminate functions among 86 mango cultivars located at RSM-collection (Rudan, Siyahu and Minab).

with low overlap. Moreover, the results of confusion matrix for the cross-validation and Mahalanobis distance based on qualitative traits indicated that the 82.55% (71 number) of total cultivars belonged to their original classes and the rest cultivars (17.44% = 15 number) were distributed among the three classes (Table S4). Overall, result of LDF on quantitative traits for interpreting the genetic relationship among cultivars was better than LDF on qualitative traits.

4. Discussion and Conclusions

This study is the first assessment of the Iranian mango germplasm focused mainly the local cultivars. In this study, the cultivars were diverse having significant variation for morphological and biochemical traits. Variation in fruit and leaf traits is a sign of the presence of high degree of genetic variations among cultivars. Because all cultivar presented in RSM-collection have been cultivated through seed, traits segregation among them will be the reason generated genetic diversity in fruit and leaf characters. Conversely, the traits did not show variation would probably due to the same seed source propagated these cultivars.

Descriptive analysis displayed a high variation in both quantitative and qualitative traits among mango cultivars, but variability in qualitative was greater than quantitative traits. These results of this study are in the agreement with other studies, where descriptive analysis revealed a suitable genetic variability in mango germplasm (Sennhenn *et al*., 2013; Jamil *et al*., 2015), and as well as in some other crops, such as garlic (Panthee *et al*., 2006), melon (Lotti *et al*., 2008) and sour cherry (Ganopoulos *et al*., 2016). This broad genetic variability is the foundation for applied crop breeding that allows for selection of superior cultivars. Besides, information of the descriptive results of this study will be helpful to proceed plant breeding and conservation programs in the future. In the present research, generally, the descriptive results showed that 'Senderi' cultivar as control had high value of quantitative and qualitative variables compared with all cultivars. It was considerable that the value of FrLe (5.83 cm) and FrWe (232.50 gr) for 'Ta Dorosht' cultivar displayed closest or same value with controls, while the high value in TSS (19 Brix) for 'Jamali-1' and in ToSu (21.92%) for 'Shahani' was observed compared to controls.

Quantitative traits are the most significant traits of the majority of plants that are mainly influenced by the environment and hence have low heritability. Because of the response to direct selection for these traits may be unpredictable, therefore, plant breeders prefer to select for related traits that indirectly increase quantitative target traits. It was obvious that most significant Pearson's correlations were obtained by phenotypic traits particularly for the fruit yield and quality. In the breeding materials, the higher variation is the greater scope for its improvement through selection. For instance, cross combinations could be performed between cultivars with very large fruit length ('Senderi', 'Khiyar-1', 'Havij' and 'Mashk'), low stone weight ('Kozekasbi', 'Shahani' and 'Zapak') and high total soluble solids ('Jamali-1', 'Houz-1' and 'Kalak sorkh-3'). In order to achieve high yield and superior quality cultivars, output information of the morphological investigations might be quite helpful for any future mango breeding in RSM-collection. The 'Jamali-1', 'Houz-1', 'Kalak sorkh-3' and 'Shahani' will be more suitable cultivar for the fresh consumption cultivars because it showed the favorable level of soluble solids and titratable acidity. In open canopy trees light could penetrate well into the canopy causing increase of photosynthesis rate and transfer of carbohydrates from the leaves to the fruits. Similarly, Farrokhi *et al*. (2013) also showed the importance the correlation of suitable canopy volume and transferred carbohydrate to increase fruit weight in apple. This aspect will be very good option in selecting the candidate mango cultivar with suitable canopy volume and fruit quality traits, such as 'Ta Dorosht', for providing a source of material breeding. TSS is an important biochemical factor that its level is increased simultaneously with fruit development (Zarbakhsh *et al*., 2020). Pearson's correlations revealed that the cultivars with higher fruit weight tend to reveal relatively higher soluble solids. This is in agreement with the previous reports in apple (Farrokhi *et al*., 2013) and in sour cherry (Ganopoulos *et al*., 2016). It was considerable that no correlation was observed between soluble solids and titratable acidity, which has been confirmed previously in apricot (Ruiz and Egea, 2008).

In breeding programs, PCA-biplot is an important tool to identify and rank the superior cultivars and thus facilitating the mango selection process (Maia *et al*., 2016). PCA-biplot for quantitative variables showed that the highest PC1 values corresponded to 63 ('Ta Dorosht') with high fruit weight, fruit length and fruit breadth which was closeted to 36 ('Senderi').

Germplasm collection and conservation are important not only to preserve genetic resources, but also to enable breeders to exploit the genetic and phenotypic variation and develop superior cultivar. Therefore, the 'Ta Dorosht' could be introducing as superior cultivar and may be used as parent in backcrossing method with common cultivars. Moreover, regarding the 27 ('Kalanfar-1') and 7 ('Majlesi-1'), it showed lower values in all variables analyzed particularly for the canopy diameter and pH values, as shown in figure 2A (were positioned in the green line triangle). It seems that above-mentioned cultivars are exposed to highly endangered in the RMS- collection and should be consider a suitable conservation program to protect the total loss of them. Also, due to low vegetative vigor and tree size, these cultivars may be useful for the breeding program and being desirable as dwarfing rootstocks. Overall, the results of PCA for quantitative traits showed a good performance regarding fruit traits such as fruit weight, fruit length and fruit breadth, which are the most important for discriminating pomological traits, while it did not indicate distinct grouping in our studied cultivars. Additionally, our results are in accordance with the previous studies, which also documented that the weight and fruit size are useful parameters to discriminate cultivars in inter-specific almond × peach (Yaghini *et al*., 2013), and in sour cherry (Khadivi-Khub *et al*., 2013; Ganopoulos *et al*., 2016). The high negative for PC1 and PC2 for qualitative traits resulted in formatting a distinct group cultivar with strong adherence in fruit, broadly pyramidal inflorescence shape, sinus fruit, and light green leaf, which were positioned in the green line trapezoid. These findings are in agreement with various other studies that reported the maximum contribution of fruit and flower traits towards the genetic divergence in cherries (Khadivi-Khub *et al*., 2013; Ganopoulos *et al*., 2016) and mango (Maia *et al*., 2016). PCA could also permit the correlation of the phenotypic traits with the genetic linkages between the respective trait's loci in QTL mapping analysis. PCA previously has been used for germplasm evaluation in almond (Nikoumanesh *et al*., 2011), apple (Farrokhi *et al*., 2013), and mango (Krishnapillai and Wilson Wijeratnam, 2016).

In cluster analysis, the highest genetic distance was detected among C2 and C3 (25.18), followed from these among C1 and C2 (19.51), and among C1 and C3 (9.25). The such dendrogram which is able to show genetic relationship among the cultivars reported by Sennhenn *et al*. (2013) in Kenya's mango and by Krishnapillai and Wijeratnam (2016) in Sri Lanka mango. It is noticed that the 'Havij' and 'Nabati-1', as the most closely related with two control cultivars was confirmed by cluster analysis and thus, they genetically were grouped together. In this study, the most closely related pairs among the mango cultivars were 68 ('Negar-1') and 74 ('Deraz') in the C2 cluster, while the highest distance was obtained for 6 ('Halow') and 86 ('Nesa-2'). Based on the results, crossing between cultivars in distanced clusters like C1 and two clusters can provide much variation for the mango breeding purposes. Parental selection in breeding program is primarily dependent on the traits desired in the progeny and is best guided by the phenotypic expression of potential parents. In dendrogram, on one side in C2 cluster, there were cultivars 77 and 75, and on the other side in C3 cluster, cultivars 42, 35, which can be recommended for parenting future crosses that could make new generations with high variations in almost all of the measured traits. Moreover, information about the similarly or dissimilarly genetic relationship among the mango cultivars could be useful for grafting compatibility and improving rootstock, where rootstock and scion share the same genetic background.

As an outcome from FA of quantitative and qualitative traits, the great variation was found in fruit morphology among cultivars. This result is in agreement with several other studies that reported high genetic diversity in mangoes (Maia *et al*., 2016). According to Martins *et al*. (2003), the variation observed in fruit morphology is very common even at intraspecific level. The variation may be attributed to environmental factors or genetic differences or both. In our study, the variations in the fruit traits can be attributed to differences in the age of the plant, fruit maturation stage, geographical sites, climatic condition, soil properties, and seed origin. Generally, this research supported that factor analysis is a useful tool for identification of the most significant variables in the biochemical and morphological data set of mangos. Factor analysis previously has been used for germplasm evaluation in several different plant (Felenji *et al*., 2011; Pour-Aboughadareh *et al*., 2017).

Discriminant functional analysis is particularly useful in defining groups of the cultivars as prior classification criteria. Moreover, it provides a graphical output illustrating the existence of groups. Generally, LDF of the variables produced better discrimination of the mango cultivars than the principal component analysis. Discriminant functional analysis previously

has been used for germplasm evaluation in several different plant (Rafiqul Islam *et al*., 2007; Sinkovič *et al*., 2017).

The present study exhibited that the presence of exploitable genetic diversity among cultivars introduced superior cultivar in which crossing of distant cultivar with desirable traits to develop cultivar for the study area and similar agro-ecology. We disclosed that many field traits have promise for Genome Wide Association Study analysis in the future, where combining molecular marker data with morphological can recognize the genes in mango controlling the main traits evaluated here. Overall, this diversity permits the effective selection of parents in various breeding programs, referring to fruit quality and aiming at different aspects of postharvest utilization, besides high yield and resistance to diseases. Hence, the present results provide important new information for the gene pool conservation, screening superior germplasm and emphasizes the importance of conservation of genetic resources for any fruit tree breeding program.

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Evaluation of hot pepper (*Capsicum* spp.) genotypes for resistance to viruses and aphids in Rwanda

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Abstract: Hot pepper is an important crop in Rwanda but viral diseases and pests are major constraints to its production. Field experiments were conducted to evaluate the resistance of 18 hot pepper genotypes (4 commercials, 5 introduced and 9 local) to natural infection by viruses and aphid infestation, in two agro-ecological zones of Rwanda. Fourteen genotypes were further evaluated for resistance to *Cucumber mosaic virus* (CMV) under screenhouse conditions. Disease incidence and severity were recorded in all experiments while population of aphids was assessed in the field. Diseased leaf samples from each genotype in the field were analysed using polymerase chain reaction to detect the presence of viruses, while samples from the screenhouse were analysed using serological assay. Results showed significant ($p<0.05$) differences in disease incidence and severity among genotypes. Three genotypes namely PBC 462, 00767PPR and 0802PPR were rated as resistant to viral diseases while genotype HP 0117, PP9852-170 and PP9950-5197 were moderately resistant. All commercial and most of the local genotypes were susceptible compared to the introduced lines. There was no difference in genotype infestation by the aphids. The genotypes that are resistant to viruses are recommended for use by growers and in breeding programs.

1. Introduction

Hot pepper (*Capsicum* spp.) is an important vegetable crop grown throughout the world. In Rwanda, it is produced for both local consumption and export to the European market (NAEB, 2015). The crop plays an important role in poverty alleviation through income generation and creation of employment to both farmers and the hot pepper value chain actors. Hot pepper production has increased in recent years in Rwanda but, the average yield is still low at around 6.8 t ha^{-1} which is lower than

50% of the country's potential yield of 15 t ha^{-1} (RDB, 2010; FAO, 2017). The low and poor quality produce have been attributed to abiotic and biotic factors, of which diseases caused by viruses play a significant role (Skelton *et al*., 2018).

Pepper is attacked by more than 68 viruses globally, of which about 15 have been identified in Africa (Njukeng *et al.,* 2013; Aliyu, 2014; Kenyon *et al.*, 2014). *Cucumber mosaic virus* (CMV), *Pepper veinal mottle virus* (PVMV), *Tobacco mosaic virus* (TMV) and *Potato virus Y* (PVY) have been reported as the most prevalent in Sub-Saharan Africa (Dafalla, 2001). In Rwanda, *Pepper vein yellows virus* (PeVYV), PVMV and CMV have been detected in hot pepper (Skelton *et al*., 2018). The CMV is ranked among the most economically important viruses of hot pepper not only in Rwanda but also in some other countries such as India where yield losses ranging from 10 to 50% are documented (Rahman *et al*., 2016). On the other hand, the crop is attacked by more than 21 insects which include aphids, whiteflies, thrips among others (Niranjanadevi *et al*., 2018). Aphids, whiteflies, and thrips are vectors of various viruses infecting hot pepper. These insect pests especially the aphids, are serious threat to hot pepper production not only due to losses caused through direct damage but also they are vectors of devastating viruses (Kenyon *et al*., 2014).

Various management options have been proposed to reduce virus diseases of hot pepper in the field. These measures include the use of virus-free planting materials, resistant varieties, borders crops, pesticides and roguing (Wang *et al.*, 2006; Degri and Ayuba, 2016). Farmers in Rwanda mainly rely on insecticides to control insect-vectors. Unfortunately, the insecticides do not achieve 100% control of the vectors. Hence, the insect-vectors develop resistance against the active ingredient after repeated application of the insecticides within a short time and more so the insecticides negatively affect the environment (Kenyon *et al*., 2014). The use of resistant cultivars offers the most economical, effective and durable solution in mitigating the negative effects due to diseases and aphids in hot pepper production (Visalakshi and Pandiyan, 2018). Resistant varieties are highly preferred because they not only reduce the pest population and the virus inoculum in the farming system but they are also compliant with other methods (Frantz *et al*., 2004). Several studies have evaluated the resistance of wild and cultivated hot pepper genotypes to virus infection and aphids' infestation leading to the release of virus-resistant lines in different parts of the world (Frantz *et al*., 2004; Appiah *et al*., 2014; Choi *et al*., 2018). However, information on hot pepper genotypes that are resistant to virus infection and vector infestation is not documented in Rwanda. The current study focused on local, commercial and introduced genotypes that have not been evaluated before for the resistance to viruses or aphids in Rwanda.

It is important to assess the genotypes in different environments in the field to identify the relative host resistance, as some genotypes found resistance at one location turns out to be susceptible to another place. Screenhouse assessment using artificial inoculation techniques is important for validation of resistance. In this study, both field and screenhouse experiments were conducted to (1) evaluate the reaction of different hot pepper genotypes (local, commercial and introduced) to natural virus infections and aphids' infestations in two agro-ecological zones of Rwanda, and (2) evaluate the reaction of selected hot pepper genotypes to infection by *Cucumber mosaic virus* under screenhouse conditions.

2. Materials and Methods

Reaction of hot pepper genotypes to virus infection and aphid infestation under field conditions

Source of seeds

A total of 18 hot pepper genotypes that included nine local collections obtained from Rwanda National Genbank, five introduced lines provided by World Vegetable Center, Eastern and Southern Africa-Tanzania and four commercial varieties from seed companies were evaluated (Table 1). Previous studies indicate that the introduced genotype PP9950-5197 is resistant to CMV, PVY and *Chilli veinal mottle virus* while genotype ICPN 18-7 is resistant to PVY (Gniffke *et al*., 2013). Similar studies by Reddy *et al*. (2014) showed the introduced genotype PP9852-170 as resistant against CMV. California wonder (sweet pepper) variety which has been used as a susceptible control for viruses in previous studies (Murphy and Bowen, 2006) was also included in the present study.

Study areas

The study was conducted at Rubona and Gashora research fields belonging to the Rwanda Agriculture and Animal Resources Development Board (RAB) during two successive growing seasons; long rains (end

March-July, 2018) and short rains (end October, 18-March, 2019). Rubona station is located at an altitude of 1692.9m, latitude S 2°28'59.59" and longitude E29°46'22.46", in midland AEZ. Gashora is located at an altitude of 1331.1m, latitude S 2°15'22.11" and longitude E30°17'12.43", in lowland AEZ. The characteristics of the two AEZs are shown (Table 2).

Raising of the seedlings

Seeds of the different genotypes were sown and raised in trays containing sterilized sandy loam soil (1:2) in the screenhouse. At 2-3 leaf stage, the seedlings were transplanted into plastic potting bags (5 × 9 × 4 cm) containing steam-sterilized sandy loam soil and maintained for six weeks in the screenhouse. Before transplanting to the field, the seedlings were confirmed to be free from *Pepper mild mottle virus* (PMMoV), PVMV, TMV, PVY and CMV using DAS-ELISA. The kits were obtained from Loewe Biochemica GmbH company, Germany) and used according to instructions from the manufacturer.

Establishment of the field experiments

The experiments were carried out in the open field and depended on natural virus infections and aphid infestation from the uncultivated fields. The experiments were laid out in a randomized complete block design (RCBD) with three replications. The blocking was done according to soil fertility gradient and nearness to the uncultivated land, such that each treatment had an equal chance of vector infestation. Each replicate had eighteen experimental plots, measuring 2.5 m by 3 m each, with a 1 m wide path between the plots. An experimental plot contained 24 seedlings planted on 4 rows, at a spacing of 60 cm by 45 cm. At planting, approximately 500 g of organic manure was used per plant and 15 g of NPK (17:17:17) fertilizer was applied one week later. A month after transplanting, 3.5 g of urea (46:0:0) per

Table 1 - List of genotypes evaluated for reaction to infection by viral diseases and aphids under field conditions in Rwanda

Genotype	Species	Type	Source
00765PPR [1]	*C. annum*	Local	RNGB
00767PPR	*C. baccatum*	Local	RNGB
00774PPR	*C. annum*	Local	RNGB
00775PPR	*C. chinense*	Local	RNGB
00786PPR	*C. annum*	Local	RNGB
00791PPR	*C. chinense*	Local	RNGB
00792PPR	*C. frutescens*	Local	RNGB
00802PPR	- [2]	Local	RNGB
00795PPR	*C. chinense*	Local	RNGB
PBC 462	*C. annum*	Introduced	WVC
PP9950-5197	*C. annum*	Introduced	WVC
HP 0117	*C. annum*	Introduced	WVC
PP9852-170	*C. annum*	Introduced	WVC
ICPN 18-7	*C. annum*	Introduced	WVC
Long Red Cayenne	*C. annum*	Commercial	Simlaw seed company
Bird-eye hybrid (Oiseau pili pili)	*C. frutescens*	Commercial	Technisem Company
Red Scotch bonnet	*C. chinense*	Commercial	Exporter
California Wonder	*C. annum*	Commercial	Kenya seed company

[1] Code of the local genotypes as found in the database of Rwanda National GenBank;
[2] Unknown species;
RNGB= Rwanda National GenBank;
WVC= World Vegetable Center.

Table 2 - Characteristics of the two agro-ecological zones of Rwanda where the study was conducted

Site/District	AEZ*	Relief	Elevation (m)	Rainfall (mm)	Temperature (°C)
Rubona/ Huye	Midlands	Dissected plateaus	1600-1900	1100-1400	17-20
Gashora/Bugesera	Lowlands	Pediplains	900-1600	850-1100	20-21

AEZ= Agro-ecological zone. Source: Verdoodt and Van Roanst, 2003.

plant was applied. Both preventive and curative fungicidal sprays were applied at regular intervals to control fungal diseases. The spray regime was dependent on symptom appearance and prevailing weather conditions. Weeding was done two times in a month. Insecticides were not sprayed at all.

Data collection

During plant growth, data was collected at a 14-days interval starting from two to ten weeks after planting (WAP) in the field. Ten plants were randomly selected from the middle rows of each plot and tagged. These plants were used for the assessment of viral disease incidence and symptom severity during the experimental period.

Determination of disease incidence and symptom severity. Virus disease incidence was expressed as a percentage based on the proportion of infected/diseased plants to the total number of plants observed per plot, as described by Galanihe *et al.* (2004).

Symptom severity was scored for the ten tagged plants in a plot based on a scale of 1-5 as described by Olawale *et al.* (2015) with slight modifications, where: 1 = no symptoms; 2 = mild symptoms of mosaic/mottling/yellowing on few leaves (<25% of the plant affected); 3 = moderate symptoms of mosaic/puckering/mottling/vein clearing/yellowing on many leaves (26-50% of the plant affected); 4 = severe symptoms of mosaic/puckering/mottling/vein clearing/yellowing/stunting (51-75% of the plant affected) and 5 = severe symptoms of mosaic/puckering/mottling/vein clearing/yellowing/ stunting/ necrosis (>75% of the plant). Percentage severity was calculated as the sum of all disease rating per genotype expressed as a percentage of the total number of observations multiplied by maximum disease scoring scale (5).

Determination of the area under disease progress curve. The area under the disease progress curve (AUDPC) was estimated to compare different responses of the tested hot pepper genotypes. Estimated percentages of symptom severity recorded at different times during the experimental period were used to calculate AUDPC using the following equation as described by Campbell and Madden (1990).

$$AUDPC\ \Sigma^{n-1} = (Y_i + Y_{i+1})/2\ (t_{i+1} - t_i)$$

where Σ = summation; n = number of successive readings, Y_i = disease severity at time t_i and Y_{i+1}= disease severity at time t_{i+1}.

Detection of the viruses. Detection of the suspected viruses in the experimental plots was carried out using RT-PCR. At 10 WAP, approximately five young leaves from diseased plants of the eighteen genotypes were collected and placed in envelopes containing silica gel. The samples were later transported to the Phytopathology Laboratory of RAB at Rubona and stored at room temperature for 4-5 days to dry. Later, the samples were grounded in liquid nitrogen to fine powders that were stored at -80°C until analyzed. In total, 68 symptomatic leaf samples were collected from Rubona and Gashora's experimental sites.

Total ribonucleic acid was extracted from 100 mg of frozen powdered hot pepper leaf tissues using acetyl trimethyl ammonium bromide (CTAB) method as described by Allen *et al*. (2006) with modifications. Amplification of CMV, PVMV, PeVYV was done using One Taq One-step RT-PCR Kit (Catalogue E531S5, New England Biolabs Inc.), following the manufacturer's instructions. Amplified products were generated using virus-specific primers that were designed during this study based on the nucleotide sequence data of CMV-R1 (GenBank accession no. MG470800.1), PVMV-R1(MG470801.1), PeVYV-R1 (MG470802.1). The CMV primers amplified a fragment of ~502 bp, PVMV a fragment of ~502 bp and PeVYV a fragment of ~498 bp (Table 3).

Thermal cycling conditions were: 48°C at 15 min

Table 3 - Primers used for detection of Cucumber mosaic virus, Pepper veinal mottle virus, and Pepper yellows virus

Virus	Primer *	Sequence	Amplification size (bp)
CMV	MG470800_1F	5'-GCTTCGCAATACGTTTTGACGG-3'	502
CMV	MG470800_1R	5'-TACGACCAGCACTGGTTGATTC-3'	502
PVMV	MG470801_1F	5'-AAGCCCTCATTGAAGGTCAACG-3'	502
PVMV	MG470801_1R	5'-ATCAACCATCACCCACATACCG-3'	502
PeVYV	MG470802_1F	5'-AGTACGTCTTCGAGACTACTGC-3'	498
PeVYV	MG470802_1R	5'-TCTATAGTAGAGAGGTCGATCC-3'	498

* Primers developed during this study.

for RT; followed by 1 min at 94°C for initial denaturation; 40 cycles of 94°C for 15 s, 54°C for 30 s and 68°C for 45 s for denaturation, annealing, and extension, respectively. The final extension was at 68°C for 5 min. These conditions were similar for the three viruses. The PCR products were separated by electrophoresis in 1.2% agarose gel stained with ethidium bromide at 100 V for 40 min in 1 × Tris-Acetate-EDTA (TAE) buffer. Gels were visualized under UV light.

Assessment of aphid population. Monitoring of aphid populations was done at a 14-days interval starting from 2nd to 12th WAP. Un-winged aphids were monitored on four plants that were randomly selected from the center of each plot. Observations were carried out on six leaves (2 upper, 2 middle and 2 lower leaves) per plant. A small camel-brush was used to dislodge and collect aphids present into small-plastic bottles containing 70% ethanol and transported to the Phytopathology Laboratory of RAB at Rubona for identification and counting. Winged aphids were captured using yellow water traps (YWT) made from yellow plastic containers that were placed in the middle of each plot and filled with 1.5 litre of tap water (Blackman and Eastop, 2000). Five millilitre of formaldehyde (10%) was added per trap to preserve the insect. The collected aphids were counted and identified to species level using existing entomological keys and stereomicroscope based on their morphological features as described by Martin (1983) and Blackman and Eastop (2000).

Reaction of hot pepper genotypes to Cucumber mosaic virus under controlled conditions

Genotypes tested

Fourteen hot pepper genotypes selected from the field trials were evaluated for resistance to CMV in the screenhouse. The experiment was carried out to validate the genotypes resistance to virus infection under controlled conditions. The genotypes tested included; seven local collections (00765PPR, 00767PPR, 00774PPR, 00786PPR, 000792PPR, 00795PPR and 00802PPR), five introduced lines (PBC 462, PP9950-5197, HP0117, PP9852-170 and ICPN 18-7) and two commercial varieties (Long Red Cayenne and Red Scotch Bonnet) as indicated in Table 1.

Inoculation of CMV

Fifty seedlings of each genotype were raised in the screenhouse. Before inoculation, the seedlings were confirmed to be free of viruses using DAS-ELISA as described in Materials and Methods. At the 5-6 leaf stage, the plants were mechanically inoculated with a local isolate of CMV. The virus was propagated and maintained in a hot pepper cultivar Scotch bonnet in the screenhouse. Infected leaves were harvested and homogenized (1: 10 w/v) in 0.1 M phosphate buffer (pH7.0) containing 0.01% of sodium sulfite. The sap was sieved to remove plant debris and 0.06% of silicon carbide was added to enhance injury and increase points of entry of the virus. Two leaves per test plant were rub-inoculated with sap extract as described by Noordam (1973). After 5 mins the inoculated plants were rinsed with distilled water to remove the excess of the inoculum. Inoculated plants were maintained in an insect free screenhouse (average 27.8°C temp, 70.8% relative humidity). In total, forty-eight plants of each genotype were mechanically inoculated with CMV. Ten healthy plants of each genotype inoculated with phosphate buffer alone (with no inoculum) were maintained as control. The symptoms development on inoculated plants were recorded up to 3 weeks' post-inoculation. At this time all the plants from the susceptible local check showed typical symptoms of CMV. The incidence and symptoms severity of CMV were evaluated on all plants as described in Materials and Methods.

Detection of CMV

The confirmation of CMV infection was performed using DAS-ELISA following the procedures described by Clark and Adam (1977) on representative samples from each hot pepper genotypes. The kits were obtained from Deutsche Sammlung Von Mikroorganismen und Zellkulturen (DSMZ, Germany) and used according to instructions from the manufacturer. A healthy sample and extraction buffer were used as negative controls. A positive control was provided with the kit. Absorbance values were read at 405 nm using a microplate reader (BioTek ELX800, USA). Due to a large number of plants, only representative samples (7) were collected from each genotype and analysed. A sample was considered positive when the absorbance value at 405 nm (A405) exceeded the mean of negative controls by a factor of two.

Classification of the hot pepper genotypes for resistance to viral diseases

The rating of the genotypes was done as described by Rahman *et al*. (2016). Based on disease incidence and severity indices for both field and

screenhouse experiments, each genotype was allocated a score of 1 to 4. Scoring for virus incidence was: <20% =1, 21-30%=2, 31-50%=3 and >51%=4. Whereas, disease severity: <1=1, 1.1-2.0=2, 2.1-3.0=3 and >3.0=4. Based on cumulative scores i.e. incidence and severity indices, the genotypes were categorized into four groups: <3= resistant (R), 4-6 = moderately resistant (MR) and 7-8 = susceptible (S). The scores for the field experiments were made on pooled data obtained from the two sites and both cropping seasons than data of individual site or season.

Data analysis

Data on disease incidence (%) and symptom severity were square-root transformed, and aphids' populations were log-transformed before subjecting to the analysis of variance (ANOVA). Means of values regarding AUDPC were worked out using the Microsoft excel program. The AUDPC values were directly subject to ANOVA. Data were analyzed using SAS statistical software program and the means were separated using the least significant difference (LSD) test at p=0.05.

3. Results

Reaction of the hot pepper genotypes to virus infection and aphid infestation under field conditions
Weather conditions at the experimental sites

The average monthly rainfall, minimum and maximum air temperature during the two cropping seasons (March-July, 2018 and October 2018-March, 2019) at Gashora and Rubona experimental sites are shown in figure 1. In Rubona, temperatures ranged from 14.4 -24.2°C in season one and 15.2-24.9°C in season two. At Gashora, there were wide variations between the minimum and maximum temperatures from 12.9-27.7°C in season one and 13.7-28.2°C in season two.

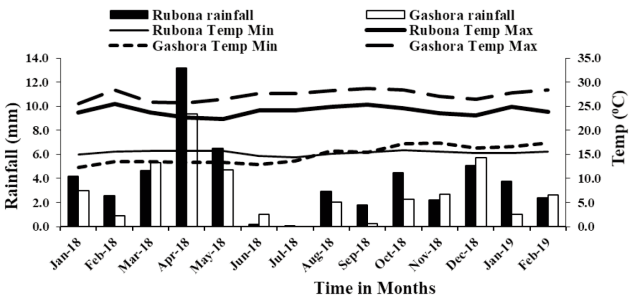

Fig. 1 - The microclimate of the experimental sites during the two cropping seasons. Temp- Temperature, Min-Minimum, Max-Maximum.

Incidence of virus diseases

There were significant differences in disease incidence between sites (p= <.0001), seasons (p= <.0001) and among genotypes (p = <.0001). The differences were observed from 4th WAP and increased with time, ranging from 3% to 100% at 10 WAP in both seasons and locations (Tables 4 and 5). At Rubona, higher disease incidence was recorded from both commercial and local genotypes, except for 00767PPR and 00802PPR compared to introduced genotypes in all sampling periods (Table 4). In season one, genotype 00767PPR was the least infected with the viruses as the disease incidence (DI) level was only 3%, followed by 00802PPR with 10%, PP9950-5197 with 20% and PBC 462 with 30% at 10 WAP (Table 4). The remaining genotypes had DI greater than 60%. In season two, disease incidence levels were generally lower in all genotypes compared to season one. The least infected genotype was PBC 462 with DI of 3% followed by 00802PPR and PP9852-170 both having 10%. This was followed by six genotypes (PP9950-5197, PBC 462, ICPN 18-7, 00767PPR, 00786PPR, 00765PPR) with incidence levels of ≤ 35% compared to the remaining genotypes that showed DI of ≥55% at 10 WAP (Table 4).

On the other hand, a similar trend was observed at Gashora, where higher disease incidence levels were recorded in season one compared to two (Table 5). Genotype PBC 462 was the least infected with DI of 13%, followed by ICPN 18-7 with 30%, PP9950-5197 with 47% and the remaining genotypes had incidence levels greater than 70% in season one (Table 5). In season two, 5 genotypes (00767PPR, PP9950-5197, PBC 462, PP9852-170 and ICPN 18-7) showed DI levels of ≤ 50%. In both sites, the highest spread of viral diseases was recorded on both commercial and local except for the genotype 00767PPR and 00802PPR. At 10 WAP, all the genotypes had developed symptoms of viral diseases but, high variability existed between genotypes. The interactions of site and season (p = <.0001), site and genotype (p = <.0001), season and genotype (p = 0.0025) were also highly significant. These results indicated that the incidence of viral diseases was dependent on the site and season the experiments were conducted.

Area under disease progress curve

The total amount of disease that occurred in the experiments was estimated and presented as the area under the disease progress curve (AUDPC). The mean AUDPC values differed significantly within sites

Table 4 - Incidence (%) of viral diseases recorded on eighteen hot pepper genotypes grown under field conditions during two seasons at Rubona Station, Huye District in Rwanda

Genotype	Type	Season one (March to June, 18)					Season two (Mid-Oct. 18 to March, 19)				
		2WAP	4WAP	6WAP	8WAP	10WAP	2WAP	4WAP	6WAP	8WAP	10WAP
00765PPR	Local [1]	0	0	43 a	97 a	100 a	0	3 b	10 bc	23 abd	33 bcdef
00767PPR	Local	0	0	0 e	3 f	3 f	0	0 b	3 c	10 bcd	17 def
00774PPR	Local	0	3	33 abc	90 ab	97 ab	0	0 b	17 ab	36 a	60 abcd
00775PPR	Local	0	10	33 abc	70 bc	87 abc	0	0 b	7 bc	20 abcd	77 ab
00786PPR	Local	0	10	37 ab	77 abc	90 ab	0	3 b	7 bc	20 abcd	30 cdef
00791PPR	Local	0	3	33 abc	80 abc	93 ab	0	0 b	0 c	23 abcd	97 a
00792PPR	Local	0	7	30 abcd	87 ab	97 ab	0	0 b	0 c	33 ab	70 abc
00802PPR	Local	0	0	0 e	3 f	10 f	0	0 b	3 c	7 bcd	10 f
00795PPR	Local	0	0	30 abcd	83 ab	97 ab	0	0 b	0 c	20 abcd	83 a
PBC 462	Introduced [2]	0	0	0 e	13 de	30 e	0	0 b	0 c	3 cd	3 f
PP9950-5197	Introduced	0	0	7 de	10 f	20 ef	0	3 b	3 c	3 cd	13 ef
HP 0117	Introduced	0	0	10 cde	37 de	63 d	0	0 b	0 c	7 bcd	13 ef
PP9852-170	Introduced	0	0	7 de	37 de	63 d	0	0 b	0 c	3 cd	10 f
ICPN 18-7	Introduced	0	0	13 bcde	40 d	63 d	0	0 b	0 c	0 d	14 f
Long red cayenne	Commercial [3]	0	0	23 abcde	77 abc	90 ab	0	3 b	10 bc	27 abcd	57 abcde
Bird eye hybrid	Commercial	0	0	7 de	40 d	70 cd	0	0 b	10 bc	33 ab	73 abc
Red Scotch bonnet	Commercial	0	3	33 abc	57 cd	80 bcd	0	0 b	10 bc	23 abd	70 abc
California Wonder	Commercial	0	0	37 ab	83 ab	100 a	0	13 a	23 a	30 abc	83 a
LSD (0.05)			10	24	26	19		5	11	28	46
P-Value			0.3699	0.0027	<.0001	<.0001		0.0002	0.0167	<.0001	<.0001

The values represent means of un-transformed data. Means comparison done by least significant difference (LSD) test on transformed data. Data transformed by square root (X+1).
Means with the same letters within a column are not significantly different (P<0.05). n= 10 replicated thrice.
WAP= Weeks after planting;
[1] Genotypes collected from farmers' field and conserved in Rwanda National GenBank;
[2] Genotypes from World Vegetable Center;
[3] Varieties obtained from seed companies and are grown for commercial purposes in Rwanda.

and thus, data were not pooled together. In Rubona, the total amount of disease was significantly ($p< 0.0001$) higher in season one with mean AUDPC values of 230.9 compared to season two that recorded 117.1. In both seasons, all commercial and local genotypes (except 00767PPR and 00802PPR) recorded high levels of disease compared to introduced genotypes (Table 6). Genotypes 00767PPR, 00802PPR, PBC 462 and PP9950-5197 consistently recorded lower AUDPC values of less than 100 in both seasons. Besides, genotypes 00786PPR, PP9950-5197, PP9852-170 and ICPN 18-7 recorded values of less than 100 but only in season two.

In Gashora, the AUDPC values were higher in both seasons compared to Rubona. However, a similar trend was observed where the amount of diseases was more in season one with values of 243.9 than season two 198.9 (Table 6). Introduced and the two local genotypes (00767PPR and 00802PPR) had low AUDPC values compared to the rest of the genotypes. Genotypes PBC 462 and PP9950-5197 recorded values of less than 100 in both seasons, while genotypes 00767PPR, PP9852-170 and ICPN 18-7 had AUDPC values of less than 100 in season two only. On the other hand, genotypes 00792PPR, 00795PPR and the four commercial genotypes were the most infected with the viral diseases in both sites during the two seasons.

Detection of the viruses in hot pepper genotypes

Three viruses were detected in the samples analyzed namely CMV, PeVYV and PVMV. *Cucumber mosaic virus* infection was the most abundant in both sites, detected in 53.1% of the samples in Rubona and 75% in Gashora, followed by PeVYV with 31.3% and 2.8%, respectively (Fig. 2). The PVMV infections were the least abundant and only detected in 21.9% of the samples in Rubona. Double infections of CMV+PeVYV were common and detected in both sites, 12.5% in Rubona and 2.7% in Gashora, followed

Table 5 - Incidence (%) of viral diseases recorded on eighteen hot pepper genotypes grown under field conditions during two seasons at Gashora Station, Bugesera District in Rwanda

Genotype	Type	Season one (March to June, 18)					Season two (Mid-Oct. 18 to March, 19)				
		2WAP	4WAP	6WAP	8WAP	10WAP	2WAP	4WAP	6WAP	8WAP	10WAP
00765PPR	Local [1]	0	0	0 d	90 a	97 ab	0	0	3	33becd	70 abc
00767PPR	Local	0	0	17 cd	40 bc	87 abc	0	0	0	0 e	10 f
00774PPR	Local	0	3	17 cd	90 a	100 a	0	0	10	63 ab	83 ab
00775PPR	Local	0	0	13 cd	77 ab	97 ab	0	0	3	13 de	53 bcde
00786PPR	Local	0	3	13 cd	100 a	100 a	0	0	0	57 bc	73 a
00791PPR	Local	0	0	17 cd	97 a	100 a	0	0	0	17 cde	83 ab
00792PPR	Local	0	7	23 abc	100 a	100 a	0	0	10	60 ab	87 ab
00802PPR	Local	0	0	0 d	43 b	63 cd	0	0	0	40 bcde	60 bcd
00795PPR	Local	0	3	17 cd	97 a	100 a	0	0	0	27 bcde	80 ab
PBC 462	Introduced [2]	0	0	0 d	0 d	13 f	0	0	0	7 efg	20 ef
PP9950-5197	Introduced	0	0	0 d	10 d	47 de	0	0	0	3 de	13 f
HP 0117	Introduced	0	0	0 d	17 bcd	70 cd	0	0	7	33 bcde	53 bcde
PP9852-170	Introduced	0	0	0 d	17 bcd	73 bc	0	0	3	23 bcde	33 cdef
ICPN 18-7	Introduced	0	0	7 cd	14 cd	30 ef	0	0	0	7 de	23 def
Long red cayenne	Commercial [3]	0	3	13 cd	87 a	100 a	0	0	10	100 a	100 a
Bird eye hybrid	Commercial	0	7	37 ab	100 a	100 a	0	0	10	43 bcd	60 bcd
Red Scotch bonnet	Commercial	0	0	20 bc	90 a	100 a	0	0	3	33 bcde	70 abc
California Wonder	Commercial	0	13	40 a	93 a	100 a	0	0	20	100 a	100 a
LSD (0.05)			9	18	29	24			13	40	39
P-Value			0.2336	0.0006	<.0001	<.0001			0.1196	<.0001	<.0001

The values represent means of un-transformed data. Means comparison done by least significant difference (LSD) test on transformed data. Data transformed by square root (X+1);
Means with the same letters within a column are not significantly different (P<0.05). n= 10 replicated thrice;
WAP= Weeks after planting;
[1] Genotypes collected from farmers' field and conserved in Rwanda National GenBank;
[2] Genotypes from World Vegetable Center;
[3] Varieties obtained from seed companies and are grown for commercial purposes in Rwanda.

Table 6 - Means of the area under disease progress curve (AUDPC) of viral diseases recorded on eighteen genotypes of hot pepper during two seasons in Rubona and Gashora sites

Genotype	AUDPC in Rubona site		AUDPC in Gashora site		Pooled
	Season one	Season two	Season one	Season two	
00765PPR	240 cdef	86 bcd	264 c	203 cdefg	197cd
00767PPR	9 h	33 cd	146 d	22 h	52 e
00774PPR	294 abc	178 ab	298 bc	334 abc	279 abc
00775PPR	281 bcd	191 ab	315 bc	129 efgh	233 bc
00786PPR	364 ab	95 bcd	311 bc	272 cde	261 abc
00791PPR	320 abc	207 ab	316 abc	191 cdefg	259 abc
00792PPR	398 a	199 ab	388 a	300 bcd	321 ab
00802PPR	21 h	34 cd	149 d	169 defgh	93 e
00795PPR	273 bcde	179 ab	333 abc	240 cdef	257 abc
PBC 462	63 gh	15 d	78 d	57 gh	55 e
PP9950-5197	62 gh	52 cd	83 d	30 h	56 e
HP 0117	165 efg	46 cd	128 d	131 efgh	117 de
PP9852-170	148 fg	27 cd	137 d	97 fgh	102 de
ICPN 18-7	179 def	16 d	93.6 d	63 gh	89 e
Long red cayenne	308 abc	146 abc	344 ab	448 a	316 ab
Bird-eye	312 abc	179 ab	346 ab	251 cde	276 abc
Red Scotch bonnet	331 abc	177 ab	350 ab	198 cdefg	264 abc
California Wonder	379 ab	248 a	312 bc	445 ab	346 a
LSD (0.05)	109.2	123.7	72.9	149.5	99.9
P-value	< 0.0001	0.0011	< 0.0001	< 0.0001	< 0.0001

The values represent means of three replicates. Means with the same letters within a column are not significantly different (P<0.05). Means comparison done by Least significant difference (LSD) test.

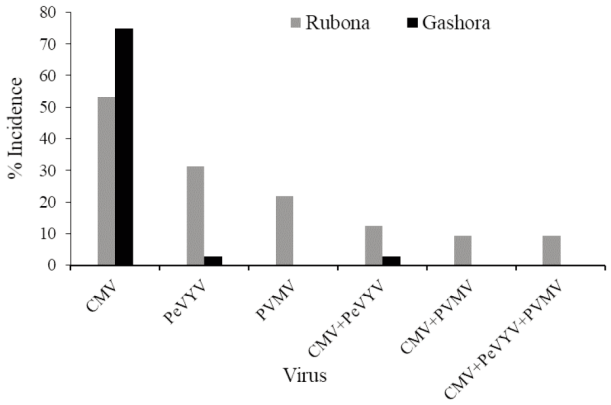

Fig. 2 - Overall incidence of viruses detected in leaf samples of hot pepper genotypes from Rubona and Gashora in Rwanda.

by CMV+PVMV detected in 9.4% of the samples tested in Rubona. Triple infection of CMV+PeVYV+PVMV was detected in 9.4% of the samples from Rubona. All hot pepper genotypes were infected by CMV.

Assessment of aphids

Aphid populations differed significantly between sites (p<0.0001), season (p=0.0075) and thus, data were analysed separately. In both seasons, the aphid population was significantly (p<.0001) higher in Rubona compared to Gashora (Table 7). Three species of aphids were observed. These were *Aphis gosypii* Glover, *Macrosiphum euphorbiae* Thomas and *Acyrthosiphon pisum* (Harris). The *A. gosypii* and *M. euphorbiae* were the most abundant in both sites, while *A. pisum* was observed in Gashora only.

All genotypes were infested by aphids but the difference in numbers were not significant (p = 0.0923) among the genotypes (Table 8). The mean number of aphids per plant ranged from 4 to 108 in Rubona and 4 to 19 in Gashora, while the mean number of aphids per leaf ranged from 0.8 to 18 and 0.6 to 3.2, respec-

Table 7 - Mean number of aphids captured in hot pepper fields during two cropping seasons in Rubona and Gashora experimental sites

Season	Rubona site			Gashora site			
	A. gosypii	*M. euphorbiae*	Total aphids	*A. gosypii*	*M. euphorbiae*	*A. Pisum*	Total aphids
Feb-June 2018	167 ± 30 a	10 ± 1 b	177 ± 30 a	32 ± 6 a	0 ± 0 b	11 ± 1 a	43 ± 5 a
Oct. 2018 -March 2019	48 ± 4 b	80 ± 11 a	128 ± 10 a	26 ± 5 a	28 ± 2 a	0 ± 0 b	54 ± 6 a
LSD (0.05)	59	21	62	16	5	2	16
P-value	0.0017	<0.0001	NS	NS	< 0.0001	<0.0001	NS

The values represent means and standard errors of three replicates.
NS= Not significant at 0.05 level.

Table 8 - Number of aphids associated with different hot pepper genotypes in Rubona and Gashora's experimental sites

Genotype	Rubona site			Gashora site			Mean aphids/plant
	Total aphids	Aphids/plant	Aphids/leaf	Total aphids	Aphids/plant	Aphids/leaf	
00765PPR	178.3	32.8	5.5	53.3	11.7	1.9	22.3
00767PPR	64.8	5.5	0.9	29	5.2	0.8	5.3
00774PPR	178.5	31.5	5.3	37.3	6.2	1	18.8
00775PPR	125.2	19.2	3.2	56	11.3	1.9	15.3
00786PPR	258.3	54.8	9	56.3	9.5	1.6	32.2
00791PPR	117.2	20.3	3.4	66.3	11.8	2	16.1
00792PPR	125.2	18.3	3	45	9.5	1.6	13.9
00802PPR	113	21.3	3.6	36.3	6.7	1.1	14
00795PPR	100.8	10.1	1.7	65.5	14	2.3	12.1
PBC 462	120	19.5	3.2	38	5.7	0.9	12.6
PP9950-5197	117.5	19.3	3.2	62.2	11	1.8	15.1
HP 0117	289.5	108	18	29.7	5.5	0.9	56.8
PP9852-170	205.8	42.2	7	94.5	19.3	3.2	30.8
ICPN 18-7	136.2	25.3	4.2	35	6.2	1	15.8
Long Red Cayenne	171	33.8	5.6	59.8	11	1.8	22.4
Bird-eye	72.2	4.8	0.8	26	4	0.6	4.4
Scotch Bonnet	83.7	10.2	1.7	50.7	9.7	1.6	9.92
California Wonder	287.8	79.2	13	40.5	7.8	1.3	43.5
P-value (0.05)	NS	NS	NS	NS	NS	NS	NS

The values represent means of untransformed data.
NS= Not significant at 0.05 level.

tively. Except HP 0117 and California wonder at Rubona site, the rest of the genotypes showed low numbers of aphids which did not exceed recommended chemical control action thresholds of 10 aphids per leaf. The population exhibited a negative correlation with minimum (r = -0.04, -0.22) and maximum temperature (0.50, -0.73) while, the correlation was positive with average rainfall (0.37, 0.68) in Rubona and Gashora, respectively. These correlations were not significant at the 5 percent level (data not shown).

Classification of hot pepper genotypes for resistance to viral diseases under field conditions

Commercial genotypes were more susceptible to virus infections than the new lines from World Vegetable Center. Various degrees of symptoms were observed on most genotypes during the evaluation period. These included leaf mosaic, crinkling, chlorosis, vein banding, and leaf deformation. Based on incidence and severity indices from both locations and seasons only five genotypes rated resistant to viral diseases i.e. 00767PPR, 00802PPR, PBC 462, PP9950-5197 and ICPN 18-7 with total scores between 2-3; three moderately resistant 00765PPR, HP 0117 and PP9852-170 with scores between 4-6; and nine susceptible 00775PPR, 00786PPR, 00774PPR, 00786PPR, 00792PPR, Long Red Cayenne, Bird Eye Hybrid, Red Scotch Bonnet, and California Wonder with scores between 7-9 (Table 9). Two local genotypes (00767PPR, and 00802PPR) and three introduced genotypes (PBC 462, PP9950-5197 and ICPN 18-7) showed resistance to viral diseases in both locations.

Reaction of hot pepper genotypes to CMV under artificial inoculation conditions

Disease incidence

A significant difference ($p<0.05$) in disease incidence and symptoms severity was observed between the genotypes tested (Table 10). Infected plants showed systemic symptoms of CMV infection including leaf mosaic, mottle, crinkling, small and deformed leaves, and stunting with varying degrees of severity (Fig. 3). Six genotypes (Red Scotch Bonnet, 00795PPR, 00792PPR, 00786PPR, 00774PPR, and Long red cayenne) developed symptoms thirteen days' post-inoculation (dpi) and the first three

Table 9 - Classification of the hot pepper genotypes based on incidence (%) and severity indices of virus-induced diseases under field conditions

Genotype	Incidence (%)				Severity indices				Cumulative rating	Host reaction
	Season one	Season two	Pooled	Rating	Season one	Season two	Pooled	Rating		
00765PPR	97	51.5	74.3 ab	4	2.4	1.6	2 d	2	6	MR
00767PPR	45	11.5	28.3 e	2	1	0.3	0.7 ef	1	3	R
00774PPR	96.5	71.5	84 a	4	2.8	2.7	2.8 abc	3	7	S
00775PPR	83.5	65	74.3 ab	4	2.6	2.2	2.4 cd	3	7	S
00786PPR	88.5	51.5	70 abc	4	2.9	2.1	2.5 bcd	3	7	S
00791PPR	90	90	90 a	4	2.9	2.7	2.8 abc	3	7	S
00792PPR	93.5	78.5	86 a	4	3.5	3	3.3 a	4	8	S
00802PPR	33	24	28.5 e	2	0.8	1.1	1 ef	1	3	R
00795PPR	91.5	81.5	86.5 a	4	2.8	2.9	2.9 abc	3	7	S
PBC 462	13	11.5	12.3 e	1	0.2	0.4	0.3 f	1	2	R
PP9950-5197	28.5	13	20.6 e	1	0.7	0.5	0.6 ef	1	2	R
HP 0117	53.5	33	43.3 cde	3	1.2	0.9	1.1 e	2	5	MR
PP9852-170	55	21.5	38.3 cde	3	1.2	0.7	1 ef	1	4	MR
ICPN 18-7	35	15	25 e	2	0.6	0.5	0.6 ef	1	3	R
Long Red Cayenne	88.5	78.5	83.5 a	4	3	3.3	3.2 ab	4	8	S
Bird-eye	76.5	55	65.8 abcd	4	3	2.5	2.8 abc	3	7	S
Scotch bonnet	80	71.5	75.6 ab	4	3	2.3	2.7 abcd	3	7	S
California Wonder	91.5	68.5	75.6 a	4	2.9	2.9	2.9 abc	3	7	S
LSD			33.6				0.7			
P-value			0.0004				<.0001			

The values represent means of un-transformed data. Means comparison done by Least significant difference (LSD) test on transformed data. Means with the same letters within a column are not significantly different ($P<0.05$). Incidence scores; 20% =1, 21-30%=2, 31-50%=3 and >51%=4. Severity scores; <1=1, 1.1-2.0=2, 2.1-3.0=3 and >3.0=4. Cumulative scores i.e. incidence + severity indices; < 3= resistant (R), 4-6 = moderately resistant (MR) and 7-8 = susceptible (S).

Table 10 - Reaction of hot pepper genotypes against Cucumber mosaic virus under screenhouse conditions

Genotype	Incidence (%)					Severity indices					Cumulative rating	Host reaction
	13dpi	15dpi	17dpi	19dpi	Rating	13dpi	15dpi	17dpi	19dpi	Rating		
00765PPR	0.0 d	0.0 d	60.4 bc	77.1 b	4	1.0 c	1.0 d	1.8 d	2.7 c	3	7	S
00767PPR	0.0 d	0.0 d	0.0 g	2.1 h	1	1.0 c	1.0 d	1.0 e	1.0 f	1	2	R
00774PPR	10.4 cd	41.7 c	41.7 cd	50.0 de	3	1.1 c	1.4 cd	2.1 d	3.1 c	4	7	S
00786PPR	4.2 cd	8.3 d	22.9 def	25 fg	2	1.1 c	1.1 cd	1.3 e	1.4 ef	2	4	MR
00792PPR	75.0 b	75 bc	75.0 ab	75 b	4	2.3 ab	2.8 b	3.2 bc	4.0 b	4	8	S
00802PPR	0.0 d	0.0 d	0.0 g	18.8 fgh	1	1.0 c	1.0 d	1.0 e	1.2 f	2	3	R
00795PPR	75.0 b	75.0 b	81.3 ab	100.0 a	4	2 b	2.7 b	3.7 ab	5.0 a	4	8	S
PBC 462	0.0 d	0.0 d	4.2 efg	12.5 gh	1	1.0 c	1.0 d	1.0 e	1.2 f	2	3	R
PP9950-5197	0.0 d	0.0 d	8.3 efg	22.9 fg	2	1.0 c	1.0 d	1.0 e	1.3 f	2	4	MR
HP 0117	0.0 d	0.0 d	2.1 fg	35.4 ef	3	1.0 c	1.0 d	1.0 e	1.5 def	2	5	MR
PP9852-170	0.0 d	0.0 d	2.1 fg	14.6 gh	1	1.0 c	1.0 d	1.0 e	1.2 f	2	3	R
ICPN 18-7	0.0 d	0.0 d	25.0 de	56.3 cd	4	1.0 c	1.0 d	1.0 e	2.0 de	2	6	MR
Long red cayenne	16.7 c	29.2 c	89.6 a	97.9 a	4	1.2 c	1.5 c	2.7 c	4.5 ab	4	8	S
Red Scotch bonnet	91.7 a	93.8 a	93.8 a	100.0 a	4	2.4 a	3.3 a	4.1 a	4.8 a	4	8	S
LSD (0.05)	12.6	15.3	21.4	19.8		0.3	0.5	0.6	0.7			
P value	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001			

The values represent means of un-transformed data. Means comparison done by Least significant difference (LSD) test on transformed data. Data transformed by square root (X + 1). Means with the same letters within a column are not significantly different (P<0.05).
Incidence scores: 20% =1, 21-30%=2, 31-50%=3 and >51%=4.
Severity scores: <1=1, 1.1-2.0=2, 2.1-3.0=3 and >3.0=4.
Cumulative scores i.e. incidence + severity indices: < 3= resistant (R), 4-6 = moderately resistant (MR) and 7-8 = susceptible (S).
n = 16 replicated three times.
Dpi= Days post-inoculations.

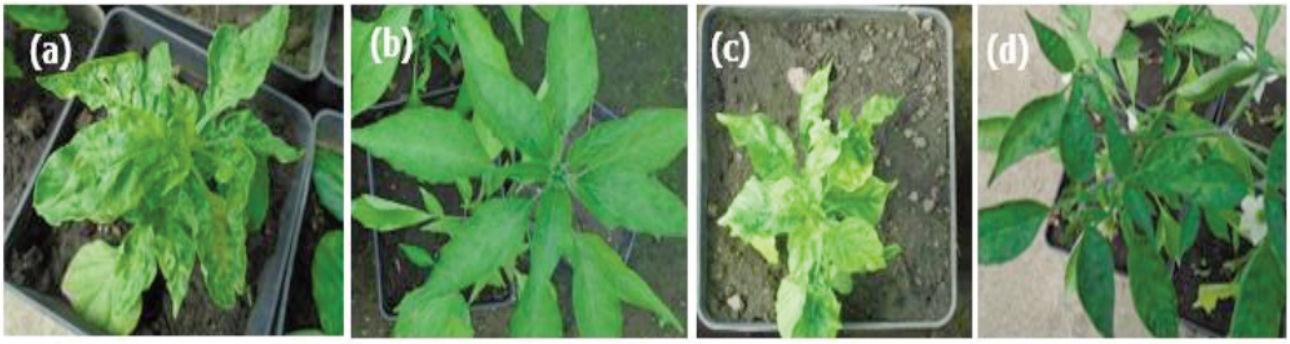

Fig. 3 - Symptoms of *Cucumber mosaic virus* on different hot pepper genotypes. (a) leaf mosaic, crinkling and distortion in commercial genotype Scotch Bonnet, (b) mottling in local genotype 00774PPR, (c) leaf distortion and stunting in local genotype 00795PPR, (d) leaf mosaic in introduced genotype ICPN18-7.

showed high levels of CMV infection (Table 10). Introduced genotypes PBC462, PP9950-5197, HP 0117, PP9852-170, ICPN18-7, and local genotype 00765PPR displayed symptoms at seventeen dpi while the remaining two local genotypes 00767PPR and 0802PPR showed symptoms at nineteen dpi. A total of six genotypes namely 00765PPR, 00792PPR, 00795PPR, 00774PPR, Long red cayenne and Red scotch bonnet had disease incidence between 50-100%, severity 2.7-5 at nineteen dpi and thus rated as susceptible to CMV (Table 10). Genotypes PP9950-5197, 00786PPR, HP 0117 and ICPN 18-7 had moderate levels of infection displaying 22-35.4% disease incidence at nineteen dpi and thus classified as moderately resistant to CMV. Among the 14 hot pepper genotypes tested, only four including two local (00767PPR, 0802PPR) and two introduced (PBC 462 and PP9852-170) showed resistant reaction against CMV with disease incidence ranging from 2-18.8% and severity 1.0-1.2 at nineteen dpi. A positive reaction to CMV was revealed by ELISA for the tested samples from all genotypes.

4. Discussion and Conclusions

Host plant resistance is an important factor in the integrated management of pests. The present study was undertaken to identify genotypes that can be used in production or as sources of resistance to viruses and aphids in hot pepper breeding programs. All the genotypes tested in this study were infected by the viruses observed either in the field or screenhouse however, there were some resistant genotypes found based on incidence and symptoms severity of the viral diseases.

In the field, five genotypes 00802PPR, *C. baccatum* 00767PPR, *C. annuum* PBC 462, PP9950-5197 and ICPN 18-7 were resistant to the viral diseases while *C. annuum* 00765PPR, HP 0117 and PP9852-170 were moderately resistant. The rest of the genotypes were susceptible to the viral diseases. These variations among the genotypes might be due to various factors that include genetic make-up, the strain of the virus and their combinations, time of infection and prevailing environmental conditions (Visalakshi and Pandiyan, 2018). Such variations reveal the diversity present within the genotypes that needs to be exploited. In previous studies under field conditions, various genotypes from *C. baccatum*, *C. annuum* and *C. frutescens* species have displayed variable resistance to some viruses such as PVMV, TMV, CMV, *Pepper mild mottle virus, Chili veinal mottle virus* and *Leaf curl virus* (Appiah *et al*., 2014; Rahman *et al*., 2016; Bandla and Beena, 2018; Fajinmi *et al*., 2018). For instance, C. *baccatum* PI 439381-1-3 was reported as resistant to CMV and PMMoV under field conditions (Suzuki *et al*., 2003). Our result, reports some additional sources of resistance from *C. baccatum* and *C. annuum* species that could be valuable in hot pepper breeding programs as well as cultivation if preferred by farmers.

The CMV, PVMV and PeVYV were detected in leaf samples collected from fields and CMV was the most abundant. These three viruses have also been reported to infect pepper previously in Rwanda (Skelton *et al*., 2018). The high incidence of CMV in the field could be attributed to several factors including wide host range, climatic conditions and efficiency of vector transmission as reported by Shah *et al*. (2009). Appiah *et al*. (2014) in their study also observed a high incidence of CMV in the range of 75% to 83.3% on pepper cultivars. All genotypes evaluated were infected with CMV and almost half of them showed multiple (double or triple) infections of CMV with either PVMV or PeVYV or both, which could have serious consequences in their management. Mixed infections of CMV and PVMV in pepper have been reported in previous studies (Aliyu, 2014; Appiah *et al*. 2014). Mixed infections in pepper plants increase the severity of disease symptoms leading to significant yield losses (Arogundade *et al*., 2012). Thus, understanding the interactions of these viruses is crucial for the development of efficient and sustainable management strategies such as resistant varieties (Syller, 2012).

Results from screenhouse showed that all genotypes developed symptoms to CMV infection albeit at different levels of severity and time, confirming the virulence of the local CMV isolate used. The plants developed systemic symptoms including mosaic, mottle, leaf crinkling and distortion and stunting which were similar to symptoms described by Rahman *et al*. (2016). Two local genotypes 0802PPR and *C. baccatum* 00767PPR, and two introduced genotypes *C. annuum* PBC 462 and PP9852-170 were found resistant to CMV while one local *C. annuum* genotypes 00786PPR and three introduced genotypes PP9950-5197, HP 0117 and ICPN 18-7 were moderately resistant. The previously published resistant genotypes (PP9852-170 and PP9950-5197) to CMV in screenhouse conditions were also resistant in our study (Gniffke, *et al*., 2013; Reddy, *et al*., 2014). However, genotype ICPN 18-7 that was previously reported as susceptible to CMV was moderately resistant in the current study (Gniffke, *et al*., 2013). The reason for these differences could be attributed to the use of different strain of CMV. Various sources of resistance to CMV in pepper have been identified in *C. annuum*, *C. baccatum* and *C. frutescens* species (Grube *et al*., 2000; Chaim *et al*., 2001; Caranta *et al*., 2002; Suzuki *et al*., 2003; Rahman *et al*., 2016). The present findings prove that natural resistance or tolerance exists in tested *C. annuum* and *C. baccatum* genotypes. As different strains of CMV exist, it is desirable to test the identified pepper genotypes against multiple strains of CMV to validate their resistance.

In both field and screenhouse experiments, genotype 00767PPR, 0802PPR and PBC 462 were consistently resistant to viral diseases while genotype HP 0117 was moderately resistant, providing evidence that the reactions of these genotypes to the virus might be due to genetic factors. However, unlike under field conditions where genotypes PP9950-5197 and ICPN 18-7 were categorized as resistant to viral

diseases, they reacted differently when subjected to the artificial inoculation with CMV and grouped as resistant. This might be due to disease escape in the field. Similar observations were made by Ashfaq *et al*. (2014), where two chili genotypes C-7 and C-8 showed a different reaction to CMV under controlled and uncontrolled conditions. On the other hand, genotype PP9852-170 was resistant to CMV under controlled conditions while in the field, it was grouped as moderately resistant to viral diseases. This may be due to the complex nature of the viruses' infection in the field where more than two viruses occur in combination. As was evident in this study where single and mixed infections of CMV, PVMV, and PeVYV were observed and their presence might have contributed towards variations in the reaction of the host in the field. These variations in the observation may also be due to variations in inoculum load and environmental conditions that might have interfered with plant behaviour.

The categorization of genotypes into resistant, moderately resistant and susceptible was based on the incidence and severity of the viral diseases on the host. However, it is noteworthy that the genotypes 00802PPR, 00767PPR, PBC 462, PP9950-5197 and ICPN 18-7 classified as resistant to viral diseases had the lowest AUDPC values of less than 100 in the field while the highest AUDPC value was recorded in susceptible check California wonder 346. Lower AUDPC values indicate a lower disease development rate. These genotypes had low AUDPC values which implies that the plant defence mechanism against the viruses could be mediated by resistance (R) genes which are observed as complete resistance or extreme resistance (ER) and that the virus replication could have been hindered or gone undetectable among the infected cells (Ingvardsen *et al*., 2010). The reaction of pepper cultivar to the viral diseases is governed by the resistance genes which can be brought by a single gene or multiple genes (Kang *et al*., 2010; Kim *et al*., 2017). However, genes responsible for their resistance in particular for the two local accessions are unknown and mechanisms that underlie their resistance are yet to be understood. This is important information that could help to determine useful markers to support breeding processes.

Aphid species are important agricultural pests because they have a broad host range and transmit many important plant viruses. In this study, three species of aphids were recorded in the pepper fields and the most abundant in both sites were *A. gosypii* and *M. euphorbiae*. These findings agree with previous studies by Meena *et al*. (2013) and Rajput *et al*. (2017) who reported the infestation of hot pepper fields with *A. gosypii* in India. Similar results on *M. euphorbiae* were reported by Djieto-Lordon *et al*. (2014) in Cameroon. The presence of *A. pisum* in Gashora was understandable since there was a pigeon peas field near the experimental plots. These polyphagous insects belong to the *Hemiptera* order and they are important pests because of the ability to transmit several viruses in pepper. According to Fajinmi *et al.* (2011); Dombrovsky *et al.* (2010) and Zitter and Murphy, (2009) *A. gosypii* efficiently transmits CMV, PeVYV, and PVMV which were detected in this study.

There was no difference in genotypes infestation by the aphids. Besides, complete genotype resistance to aphids' infestation was not recorded in any of the genotypes tested. Bird-eye hybrid was the less preferred by the aphids (4.4 aphids/plant) followed by 00767PPR (5.3 aphids/plant) and Red scotch bonnet (9.9 aphids/plant) while the most preferred was genotype HP 0117 (56.8 aphids/plant) followed by California wonder (43.5 aphids/plant) and 00786PPR (32.2 aphids/plant). Unlike other plant species such as soybean where a lot has been done on resistance to aphids (Hill *et al*., 2004), only a few studies have been conducted on pepper (Frantz *et al*., 2004; Sun *et al*., 2018). Sun *et al*. (2018), in their studies, identified *C. baccatum* accession PB2013071 as highly resistant, while the accessions PB2013062 and PB2012022 as intermediate resistant to *M. persicae* under screen house conditions. Recently, quantitative trait loci (QTLs) conferring resistance to *M. persicae* in pepper was detected (Sun *et al*., 2019). In the present study, prevailing weather conditions, especially at the Gashora site, negatively affected the population of aphids leading to low infestation on pepper plants. Thus, further efforts are needed to identify and validate the resistance of these genotypes to aphids under controlled and uncontrolled conditions.

Most of the varieties grown in the country including the commonly grown commercial varieties were found to be susceptible to viral diseases. A relatively higher number of resistant lines from introduced material indicates that the World Vegetable Center germplasm collection has a wider genetic base than local material. Since viruses cause serious diseases of hot pepper around the world, the results of this study may be promising and could be used in the for-

mulation of integrated control strategies for the management of these destructive 1diseases. The use of resistant pepper genotypes to manage the viral diseases can potentially replace or minimize the application of harmful pesticides and could be used as an important component of integrated pest management (IPM) which is a promising approach to sustainable agriculture.

In the present study, three genotypes 00767PPR, 00802PPR and PBC 462 consistently rated as resistant to viral diseases while genotype HP 0117, PP9852-170, PP9950-5197 and ICPN 18-7 were moderately resistant under field and screenhouse conditions. As revealed from the study, most of the local genotypes and all of the commercially grown pepper genotypes tested were susceptible. Therefore, the identified genotypes especially the ones from World Vegetable Center are recommended for adoption by growers. The two local collections 00767PPR and 00802PPR are not preferred cultivars for commercial production and thus, they can be utilized in breeding programs as potential sources for virus resistance. Farmers should be encouraged to use hot pepper varieties that are resistant to viruses as part of a management program to maximize yields. Further studies are needed to identify and validate resistance of the tested genotypes to aphids under controlled and uncontrolled conditions.

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Identification and impact of phytoplasmas associated with greenhouse cucumber phyllody in Iran

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Key words: *Cucumis sativus*, PCR, RFLP, 16SrVI-A, 16SrXII-A.

Abstract: Cucumber phyllody symptoms were observed in greenhouse cucumber plants during 2014-2018 in all surveyed areas of central and west of Iran where the highest disease incidence was up to 82% in Taft (Yazd province). Symptoms exhibited by diseased plants were virescence, phyllody and sterility of the flowers. For verification of phytoplasma presence and identity, total DNAs were extracted from 44 symptomatic and six asymptomatic plants that were subjected to PCR amplifying 16S rRNA genes of phytoplasmas. PCR amplicons of the expected size were obtained only from the symptomatic plants. RFLP analysis of R16F2n/R2 amplicons showed patterns identical to those of the clover proliferation (16SrVI) and "stolbur" (16SrXII) phytoplasma groups. Consensus sequences corresponding to phytoplasma strains from the two localities Taft and Shahrekord showed 99% identity with phytoplasmas enclosed in groups 16SrVI and 16SrXII, respectively. Phylogenetic analysis confirmed that these phytoplasmas cluster with '*Candidatus* Phytoplasma trifolii' and '*Ca*. P. solani', respectively. Virtual RFLP provided profiles identical to the patterns of 16SrXII-A and 16SrVI-A phytoplasma subgroups. These phytoplasma subgroups were previously reported in different plant species growing near to the greenhouse cucumber areas in Iran, and play a possible role in the epidemiology of disease for its dissemination.

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1. Introduction

Among the cucurbitaceous plants grown in greenhouses, *Cucumis sativus* with 7,427 ha is considered the most economical important crop in Iran where about the 77% of the area under greenhouse cultivation is greenhouse cucumber (Iranian Ministry of Agriculture, 2019). These plants need less water than the species cultivated in the fields, and due to the water constraint, this production is expanding. The presence of phyllody disease was reported in cucumber up to 80% in Jiroft and Kahnooj

(Kerman province, Iran) (Azadvar *et al.,* 2004). In some areas, due to high disease incidence and severity, infected plants did not bear fruits and farmers remove cultivated cucumbers and re-sown them. In 2004-2006 surveys in greenhouses the presence of cucumber phyllody was observed in Yazd, Varamin, and Larestan with about 35%, 80% and 3% of disease incidence respectively, and the phytoplasma presence was confirmed (Esmaeilzadeh-Hosseini *et al*., 2006). Phytoplasmas are destructive bacteria infecting more than one thousand plant species worldwide. They are transmitted mainly by leafhoppers and symptoms include yellowing, discoloration, dwarfing, witches' broom, virescence and phyllody (Bertaccini *et al.,* 2014). Their identification relies on molecular classification based on the amplification and/or RFLP analyses of their 16S rRNA gene (Lee *et al*., 1998; IRPCM, 2004). The aim of the present work was to identify the phytoplasmas associated with greenhouse cucumber phyllody in central and west parts of Iran in order to devise the appropriate disease management to reduce its economic impact.

2. Materials and Methods

During 2014 to 2018, greenhouse cucumber growing areas of central and west of Iran were surveyed for evaluation of phytoplasma disease presence. Symptomatic and symptomless cucumber plants were collected in greenhouses located in Akramia, Chah Shahrdar and Taft (Yazd province) and Shahrekord region (Chaharmahal and Bakhtiari province) and subjected to molecular studies for phytoplasma detection and identification. Sampling was carried out randomly in five 1,000 m^2 greenhouses and the disease incidence was calculated by counting the number of symptomatic plants exhibiting phyllody out of the total number of greenhouse cucumber plants in each greenhouse multiplied by 100.

Nucleic acid extraction was carried out as described by Zhang *et al.* (1998) using 0.2 g of fresh midrib tissue from 44 symptomatic greenhouse cucumber plants and from 6 asymptomatic seed grown greenhouse cucumber plants. The DNA from pot marigold phyllody phytoplasma (16SrII-D subgroup) (Esmaeilzadeh-Hosseini *et al.,* 2018) was used as positive control. A total of 100 ng of nucleic acid was used for the PCR to amplify the 16S rRNA gene of the phytoplasmas with primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.,* 1995) followed by nested PCR using R16mF2/R16mR2 and R16F2n/R16R2 (Gundersen and Lee, 1996) primers in a total volume of 50 µl. One µL of the products from direct amplification was diluted in 29 µL of sterile deionized water for the nested amplifications. The PCR reaction was performed in 50 µL mixtures containing 0.4 µM of each primer, 0.2 mM of each dNTP, 1.25 U Taq DNA polymerase and 1X Taq polymerase buffer (CinnaGen, Iran). PCR protocols were done as reported by Salehi *et al*. (2015). Following PCR, 2 µL of each reaction mixture was electrophoresed in a 1% (w/v) agarose gel containing 0.3 µg/mL ethidium bromide in 0.5 X TBE buffer (22.5 mM Tris-borate, 1 mM EDTA, pH 8.0). The amplicons obtained with R16F2n/R16R2 primers were analyzed by single restriction endonuclease digestion with *Alu*I, *Hae*III, *Taq*I, *Hpa*I, *Hpa*II, *Mse*I, *Rsa*I, *Kpn*I and *Hha*I (Thermo Scientific). The digestion products were analyzed through an 8% polyacrylamide gel electrophoresis and the visualization of DNA bands was carried out with a UV transilluminator after staining by ethidium bromide.

Direct sequencing in both directions of twelve samples (three per each greenhouse area) was carried out using R16mF2/R16mR2 amplicons and the same primers. The resulting sequences were trimmed to the R16F2n/R2 fragment (about 1,240 bp) and submitted to GenBank. A database search of homologous sequences was performed by BLAST analyses at the NCBI (www.ncbi.nlm.nih.gov). The R16F2n/R2 sequence of the 16S rRNA gene of greenhouse cucumber phyllody phytoplasma strains SGCP (Shahrekord greenhouse cucumber phyllody), TGCP (Taft greenhouse cucumber phyllody), Fars (GenBank accession number JN574839) and Tehran (GenBank accession number MH004460) and of 16S rRNA gene of selected '*Candidatus* Phytoplasma' species or phytoplasma strains enclosed in the subgroups of groups 16SrVI and 16SrXII were aligned (Table 1). A phylogenetic tree was constructed with the phytoplasma sequences obtained and others retrieved from the GenBank using the neighbor-joining method with MEGA software version 7 (Kumar *et al.,* 2016). *Acholeplasma laidlawii* was used as an out-group to root the tree and bootstrapping was performed 1,000 times to estimate the stability and support for the branches. The ribosomal subgroup affiliation of the detected phytoplasmas was confirmed by virtual RFLP analysis with the *i*Phyclassifier (Zhao *et al.,* 2009).

Table 1 - Phytoplasma sequences used for comparison with the reported greenhouse cucumber strains from Iran

Disease or phytoplasma	GenBank accession number	Country	16S ribosomal subgroup
'*Ca*. P. trifolii'	AY390261	Canada	16SrVI-A
Strawberry multiplier disease	AF190224	Canada	16SrVI-B
Illinois elm yellows	AF409069	USA	16SrVI-C
Periwinkle little leaf	AF228053	Bangladesh	16SrVI-D
Centaurea solstitialis virescence	AY270156	Italy	16SrVI-E
Catharanthus phyllody	EF186819	Sudan	16SrVI-F
Portulaca little leaf	EF651786	India	16SrVI-H
'Ca. P. sudamericanum'	GU292081	Brazil	16SrVI-I
'Ca. P. solani'	AF248959	Serbia	16SrXII-A
'Ca. P. australiense'	L76865	Australia	16SrXII-B
Strawberry lethal yellows	AJ243045	Australia	16SrXII-C
'Ca. P. japonicum'	AB010425	Japan	16SrXII-D
'Ca. P. fragariae'	DQ086423	Lithuania	16SrXII-E
"Bois noir" strain BN-Op30	EU836652	Italy	16SrXII-F
"Bois noir" strain BN-Fc3	EU836647	Italy	16SrXII-G
'Ca. P. convolvuli'	JN833705	Italy	16SrXII-H

3. Results and Discussion

Greenhouse cucumber diseased plants showed flower virescence, phyllody and sterility (Fig. 1), the disease was named greenhouse cucumber phyllody (GCP).

The symptoms were observed in all the greenhouses of the four surveyed areas. The disease rate was up to 11.2%, 33.5%, 82% and 8.7% in Akramia, Chah Shahrdar, Taft (Yazd province) and Shahrekord region (Chaharmahal and Bakhtiari province), respectively.

PCR amplicons of about 1.8, 1.4 and 1.25 kb were obtained from all the symptomatic greenhouse cucumber samples but not from the symptomless ones. Restriction fragment length polymorphism (RFLP) analysis of R16F2n/R16R2 amplicons using *Alu*I, *Hae*III, *Taq*I, *Hpa*I, *Hpa*II, *Mse*I, *Rsa*I, *Kpn*I and *Hha*I restriction enzymes showed two RFLP pattern identical to those reported for the 16SrVI and 16SrXII phytoplasma groups, respectively (Fig. 2).

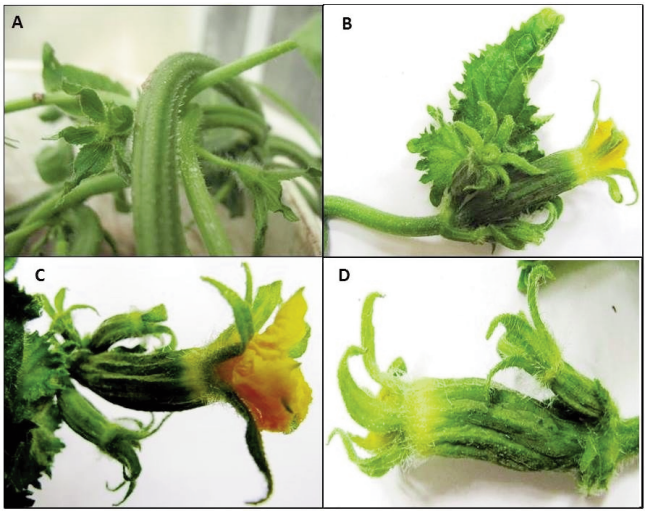

Fig. 1 - Flower virescence, phyllody and sterility in a greenhouse cucumber plant from Yazd (A) and Chaharmahal and Bakhtiari (B, C, D) provinces.

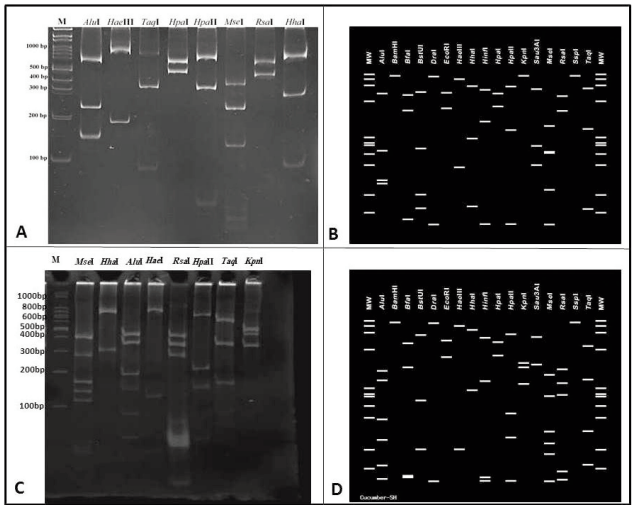

Fig. 2 - Real and virtual RFLP patterns (Zhao *et al*., 2009) respectively of 1.2 kb amplicons from TGCP (A and B) and SGCP (C and D) phytoplasma strains 16S ribosomal gene sequence. Lane M, 100 bp DNA ladder (Biobasic, Canada). DNA products were digested with the enzymes listed at the top of the figures.

The DNA fragments obtained from twelve samples after direct sequencing were aligned and the consensus sequences corresponding to a representa-

tive of GCP phytoplasmas in Taft (TGCP) and Shahrekord (SGCP) were deposited in GenBank under the accession numbers MF438041 and MK402983, respectively. The BLAST search of these sequences showed that TGCP (1,251 bp) and SGCP (1,243 bp) phytoplasmas had 99.60% and 99.28% identity with phytoplasmas enclosed in subgroups 16SrVI-A ('*Ca*. P. trifolii', GenBank accession number AY390261) and 16SrXII-A ('*Ca*. P. solani', GenBank accession number AF248959), respectively. The phylogenetic analysis confirmed that TGCP phytoplasmas cluster with phytoplasmas classified in the 16SrVI group and were therefore confirmed as closely related to '*Ca.* P. trifolii', while the SGCP phytoplasmas cluster with those enclosed in the 16SrXII group and were therefore related to '*Ca*. P. solani' (Fig. 3). The R16F2n/R2 amplified regions from TGCP and SGCP phytoplasmas digested *in silico* with 17 restriction enzymes

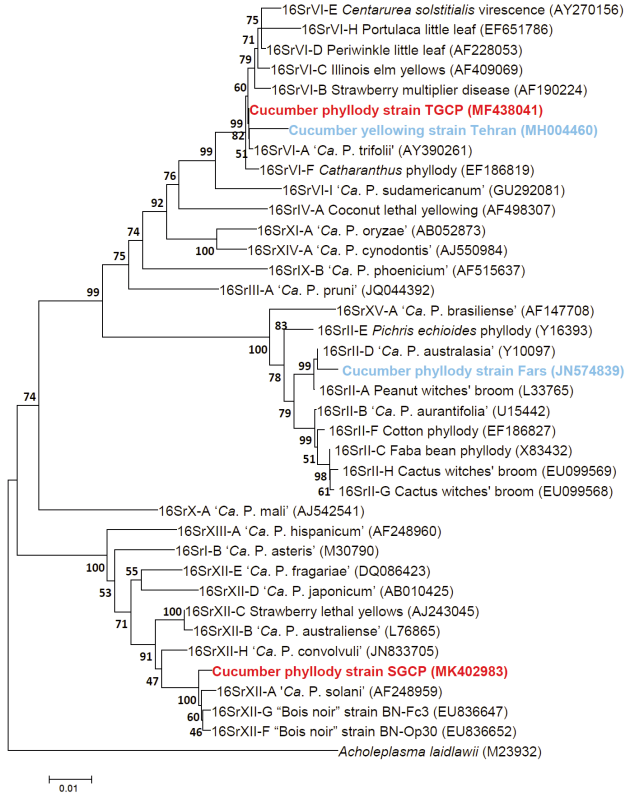

Fig. 3 - Phylogenetic tree constructed by the Neighbor-Joining method of the R16F2n/R16R2 sequence of 16S rRNA gene of 38 phytoplasmas including the cucumber strains SGCP, TGCP, Tehran and Fars, and phytoplasmas enclosed in subgroups of the 16SrVI, 16SrII and 16SXII groups. The greenhouse cucumber phyllody phytoplasmas are in color and bolded (in red those sequenced in this work). Numbers at the nodes are bootstrap values based on 1,000 repetitions. 'Ca. P.': 'Candidatus Phytoplasma'. GenBank accession numbers for sequences are given in parentheses while the phytoplasma ribosomal grouping is before the strain name.

using online *i*PhyClassifier program exhibited virtual RFLP profiles identical to the reference pattern of 16SrVI-A and 16SrXII-A, respectively (Fig. 2).

Phytoplasmas belonging to diverse ribosomal groups have been detected in *Cucurbitaceae* species worldwide showing different arrays of symptoms. In particular, 16SrI in *Cucurbita pepo* L. in Italy (Minucci *et al.,* 1995) and in *Sechium edule* (Jacq.) Sw. in Costa Rica (Villalobos *et al.,* 2002), 16SrII in *C. sativus* and *C. pepo* in Australia, Egypt and Iran (Davis *et al.,* 1997; Omar and Foissac, 2012; Salehi *et al.,* 2015), 16SrIII in *Luffa cylindrica* L. (Rox.) and *Sicana odorifera* (Vellozo) Naud in Brazil (Montano *et al.,* 2000, 2007a, 2007b) and 16SrVIII in *L. cylindrica* in Taiwan (Davis *et al.,* 2017).

Phyllody is an important phytoplasma disease of cucurbitaceous plants in Iran (Salehi *et al.,* 2015) and it was associated with the presence of a peanut witches' broom phytoplasma (16SrII) in greenhouse cucumber plants showing phyllody (Dehghan *et al.,* 2014) and of a clover proliferation phytoplasma (16SrVI) in Tehran (Ghayeb Zamharir and Azimi, 2019). Molecular assays confirmed the phytoplasma presence in the symptomatic greenhouse cucumber analyzed in this work and allow their identification as '*Ca*. P. trifolii' (16SrVI-A) and '*Ca*. P. solani' (16SrXII-A)-related strains (Esmaeilzadeh Hosseini *et al*., 2019).

In the present work the identification of phytoplasmas in subgroups 16SrVI-A and 16SrXII-A allows epidemiological considerations. The 16SrVI-A-related phytoplasma strain was identified in the central areas of Iran, in the Yazd province where the most important plant species harboring 16SrVI phytoplasmas are tomato and eggplant (Salehi *et al.,* unpublished) and alfalfa (Esmaeilzadeh Hosseini *et al.,* 2015a, 2015b; Purmohammadi *et al.,* 2017). Greenhouse cucumber phyllody associated with the presence of 16SrXII-A ("stolbur") phytoplasmas was present in Chaharmahal and Bakhtiari province where this phytoplasma was detected also in grapevine showing diverse symptoms (Mirchenari *et al.,* 2015) and alfalfa showing witches' broom (Esmaeilzadeh Hosseini *et al.,* 2016a, 2016b). Phytoplasma diseases associated with "stolbur" were present in plant host species adjacent to greenhouses cucumber areas but their role in the epidemiology of the disease needs to be proved.

Due to the problems of water constraint, the cucumber greenhouse cultivation in Iran has been widely increased. In the majority of cucumber production greenhouses, aeration valves are usually not

Fig. 4 - Aeration valves are usually not covered with netting by greenhouse owners and this allows the entry of insect vectors.

covered with netting by greenhouse owners (Fig. 4) which allows also the possible entry of insect vectors. Furthermore, the major cucumber greenhouses are located close to the agricultural fields and rangelands where during the recent droughts, the insect vectors are attracted and possibly transmitted the phytoplasmas from sources outside the greenhouse.

It is therefore probable that infection in these plants play a role in the epidemiology for the dissemination of this bacterium in the greenhouses also considering that the plants are not completely isolated from the environment. The presence of consistent populations of *Orosius albicinctus* and *Circulifer haematoceps* both recognized vectors of the cucumber phyllody disease was detected in plants grown adjacent to these greenhouses (Salehi *et al*., 2015). Their feeding activity during the year, especially in the Yazd province, leads to the widespread dissemination of phytoplasmas; therefore, preventing the entry of insect vectors into the greenhouses is the most recommended management to reduce the disease incidence.

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AHS
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Growth and chlorophyll fluorescence characteristics of *Sinningia speciosa* under red, blue and white light-emitting diodes and sunlight

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Key words: Greenhouse, growth chamber, light-emitting diodes, light spectra, transplant production.

Abstract: Determining the most reasonable LED spectral composition wavelengths on *Sinningia speciosa* transplants was the main focus of present experiment. Seeds were sown in cell trays under chambers with distinct spectral composition including white+blue+red (WBR), blue+red (BR) and white+red (WR) LEDs with equal light quality proportions (70 µmol $m^{-2}s^{-1}$ photon flux density) and under sunlight (400 µmol $m^{-2}s^{-1}$ photon flux density) in constant conditions of 14h photoperiod, 70% relative humidity and day/night temperature of 23/18°C for 50 days. In this stage, LED treatments led to higher germination percentage and better results in biomass, canopy width, leaf width and leaf area as well as chlorophyll and carotenoids accumulation were obtained in comparison with sunlight. Extracted and technical parameters of chlorophyll fluorescence induction kinetics and maximum quantum efficiency of photosystem II (F_v/F_m) were decreased by sunlight-grown seedlings. F_v/F_m was induced by WBR and BR treatments, correlated with maximum yield of primary photochemistry (φP_0). Quantum efficiencies (φP_0, φE_0 and ψ_0) and performance index of absorption energy flux (PI_{ABS}) were increased in BR-exposed transplants. In pot stage, LED-treated plants exhibited better results in morphological features with earlier marketable flowering stage especially under WBR, which can compensate costs of production in marketing stage.

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All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

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1. Introduction

Sinningia speciosa (Lodd.) Hiern. is a perennial potted flowering plant commonly known as Gloxinia, which is a herbaceous tropical species native to Brazil and belongs to Gesneriaceae family (Larson, 1992). Proper seasonally light adjustments are critical for production of Gloxinia, hence various source of artificial light has been effectively applied including fluorescent, high-pressure metal halide, high-pressure sodium with the optimal intensity of 45 to 70 µmol $m^{-2}s^{-1}$ (Larson, 1992; Dole and Wilkins, 2005). Even though aforementioned sources induce an increase in daily

photosynthetic flux intensity, they are not energetically efficient as desired and there is no capability of spectral manipulation. Light-emitting diodes, including diverse size, long lifetime, solid state construction (Heo *et al*., 2002; Kim *et al*., 2004), low thermal output, specific wavelength, adjustable light quality and intensity (Okamoto *et al*., 1997) and high electrical efficiency (Bula *et al*., 1991), represent a promising technology for the greenhouse industry which has technical advantages over other artificial light sources (Mitchel *et al*., 2012). Capability of LED's spectrum adjustment results in better responses of photoreceptors, influencing plant physiology and morphology and ultimately enhances production (Morrow, 2008). Horticultural crop seedlings are intensively influenced by light spectrum which affects their morphological properties (McNellis and Deng, 1995). Production of transplants under desirable light spectrum and suitable control of environmental conditions can improve transplant quality compared to traditional greenhouse production conditions in which accordingly affects their growth and yield after transplantation (Oda, 2007). Producing a large number of seedlings in a small area justifies high electricity consumption that is economically advantageous. Developing various light spectral ratio recipes for different horticultural transplants based on their demand, would influence growth rate and improve quality (Hernández *et al*., 2016), as different wavebands were proved to have significant physiological effects on plants (Kim *et al*., 2004; Johkan *et al*., 2010) and can be assembled according to the light quality which plants need (Goins *et al*., 1997).

Detecting specific optimal light spectrum prevents energy loss for physiologically none-useful wavelengths (Kim *et al*., 2004; Johkan *et al*., 2010) and it can regulate a variety of plant development pathways from germination to flowering induction (Jiao *et al*., 2007).

Based on previous studies, it has been shown utilizing red (600-700 nm) and blue (400-500 nm) LEDs have the greatest impact on plant growth (Yorio *et al*., 2001) since they mainly contain range of wavelengths essential for plants photosynthesis (Cosgrove, 1981; Kasajima *et al*., 2008). Although blue light is photosynthetically less efficient than red light (McCree, 1972; Dougher and Bubgee, 2001), it has considerable photomorphogenic effects on chlorophyll biosynthesis, stomatal opening, enzyme synthesis, photosynthetic capacity on chloroplast (Tibbits *et al*., 1983), fresh and dry matter accumulation, flowering (Withelam and Halliday, 2007; Johkan *et al*., 2010), stem elongation and leaf expansion (Hoenecke *et al*., 1992; Dougher and Bubgee, 2001).

Researches have demonstrated that combinational light regimes may help to optimize growth (Brown *et al*., 1995). Various number of studies have suggested that combination of red, blue and white LED lighting in different ratios is a favorable lighting condition for plants in many aspects (Lin *et al*., 2013; Ouzounis *et al*., 2014). Combination of red-blue, red-white, red-blue-white provides the highest photon efficiency as compared to monochromatic LED illumination (Lin *et al*., 2013; Nelson and Bubgee, 2014; Ouzounis *et al*., 2014; Nicole *et al*., 2016). Few studies on continuous-spectrum LED lamps fit to a theoretical model of the maximum photosynthetic response has been recorded since McCree's (1972) experiments on cultivated plants.

It has been shown that chlorophyll fluorescence data can help with analyzing energy flow and information related to the structure and function of photosynthetic apparatus (Brestic and Zivcak, 2013). The non-destructive analysis of polyphasic fast chlorophyll transient by the so-called OJIP test was developed for quick evaluation of biophysical aspects of photosynthesis (Strasser, 1995; Mathur *et al*., 2013). This test which is based on energy flow in thylakoid membranes provides detailed information about the biophysics of the photosynthetic system through measurement of fluorescence signals (Kalaji *et al*., 2017).

The main objective of this study was to investigate different ratios of blue and white with the red spectral composition (WR, BR, WBR) to determine the most effective combination of waveband in comparison with natural light condition.

2. Materials and Methods

Plants material and lighting treatments

This experiment was carried out in 2018-2019 in specialized chambers in Shahrekord University research greenhouses. *Sinningia speciosa* F1 (brocade blue) pelleted seeds were sown into 288 cell trays filled with a peat moss with the pH of 5.5-6 and EC of 1 dS/m^{-1} (1:2 dilution). Cell trays were placed inside three chambers with LED Lighting treatments and one cell tray under 50% shaded sunlight in greenhouse, as control, using a completely randomized design. Day/night temperature (23/18°C), relative humidity (70%) and photoperiod (14-hour) were maintained constant in all (LED and sunlight) treatments. Plants were grown under LED modules

(Shezhen Sunled Lighting Co., Ltd, CN Manufacturer, China) yielding approximately 70 µmol $m^{-2}s^{-1}$ measured and adjusted using PARmeter (Apogee Quantum meter, MQ500, USA). The peak emissions of blue (460 nm), red (620 nm) and white (in the range of 380-750 nm) were measured and recorded using spectrometer (BLACK-Comet CXR-SR-50, StellarNet, Inc., USA) with range of 300-800 nm (Table 1). During transplant production cell trays were exposed to 50% white + 50% red (WR), 50% blue + 50% red (BR) and 33.3% white + 33.3% blue + 33.3% red (WBR) LED and 50% shaded sunlight (SL) treatments (Table 1). The relative spectral distribution of light treatments are presented in figure 1. Cell trays were rotated frequently in order to ensure equal growth conditions. Subsurface irrigation was applied every 3 days and plants were irrigated as needed with a 500-1000 ppm water-soluble Radixol fertilizer (N:P:K + microelements; 15:17:15 + 0.12% Mg, 0.02% B, 0.0075% Cu, 0.04% Mn, 0.01% Mo, 0.012% Zn). Measurements were conducted in both transplant and flowering stages.

Transplant stage measurements

Germination and morphological characteristics. After 50 days under light treatments (10 days for germination + 40 days for growth till four fully expanded leaves observed), germination percentage was calculated. Plugs with four fully expanded true leaves, were then transplanted to 12 cm pots, in a completely randomized design with five replications (n=5). Morphological traits of five randomly selected plants from each replicate including shoot fresh and dry weight, root fresh and dry weight, leaf area, leaf width and canopy width was measured. Shoots and roots were dried in a drying oven at 72°C for 24 hours

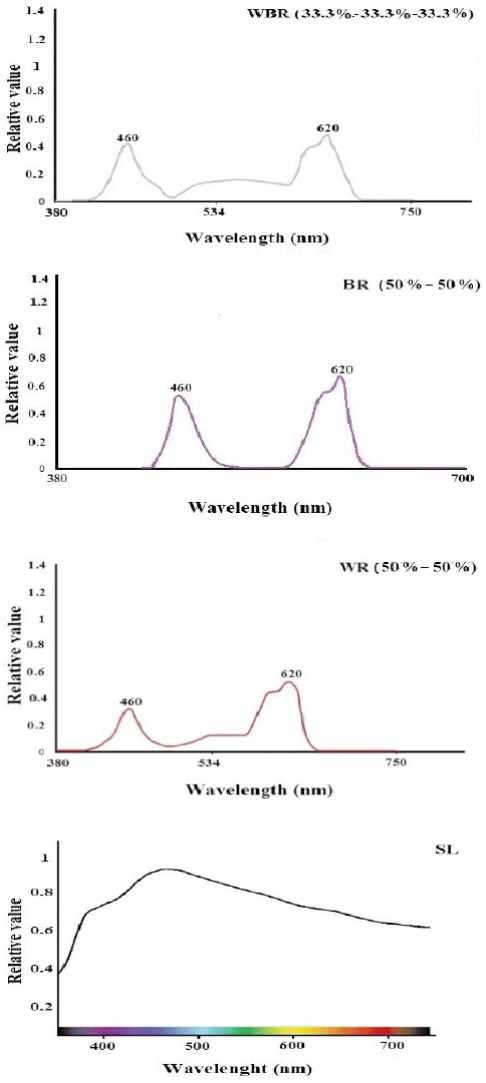

Fig. 1 - Relative distribution spectra of white+blue+red (WBR), blue+red (BR), white+red (WR) and sunlight (SL) treatments. Wavelength peaks are indicated by values above each peak.

Table 1 - Light intensity, different light spectra and light quality were used for growing *Sinningia speciosa* transplants

LED treatment	Proportion (white: blue: red)	Light intensity (mmol $m^{-2}.s^{-1}$)
WBR	33.3% : 33.3% : 33.3%	70 ± 3.1
BR	0% : 50% : 50%	70 ± 1.0
WR	50% : 0% : 50%	70 ± 2.1
Control light treatment	*Average light intensity (mmol $m^{-2}.s^{-1}$)*	
%50 Shaded sunlight (SL)	400 ± 50	
Light quality	*Light spectrum (nm)*	
White	380-750	
Blue	460	
Red	620	
SL (control)	400-700*	

*: For comparing sunlight quality with LEDs, sunlight has purple/blue : green/yellow : orange/red (400-492 : 493-597 : 598-700 nm) with the relationship of 23 : 28 : 39 according to Aphalo *et al*. (2012).

to determine dry weight. The leaf area of plants was measured by Digimizer V. 5.4.6 software.

Biochemical measurements. Biochemical measurements included chlorophyll a, chlorophyll b, total chlorophyll (a + b), carotenoid and total soluble sugar contents. Chlorophyll and carotenoid contents were extracted from 0.5 g fresh leaf tissue from five randomly selected transplants (n=5) and the pigments were eluted with 10 ml of 80% acetone centrifuged at 4000 *g* for 10 min and the amount of chlorophyll was estimated spectrophotometrically (using PG instruments T80+) at 470, 646 and 663 nm by the method of Lichtenthaler and Welburn (1983).

Total soluble sugars was quantified in 95% ethanol extracts of leaf tissue from five randomly selected transplants (n=5). A sample of 0.5 g of freshly harvested leaves was crushed in 5 ml of 95% (V/V) ethanol. The insoluble fraction of the extract was washed twice with 5 ml of 70% ethanol. All soluble fractions were centrifuged at 3500 *g* for 10 min. The supernatants were collected and stored at 4°C for TSS determination. TSS were analyzed by reacting 0.1 ml of the alcoholic extract with 3 ml freshly prepared anthrone reagent (150 mg anthrone + 100 ml 72% [W/W] H_2SO_4) and placed in a boiling water bath for 10 min. After cooling, the absorbance at 625 nm was determined (Irrigoyen *et al*., 1992) in a PG instruments T80+ spectrophotometer and the data was pooled and extracted with standard curve (y = 0.002x - 0.009, R^2 = 0.992).

Chlorophyll fluorescence and OJIP test parameters

After 50 days of growth (at four true leaves stage), a portable PAR-FluorPen FP 100 max (Photon System Instrument, PSI, Czech Republic) was used to measure maximal quantum efficiency of Photo System II (F_v/F_m) photochemistry. The most recent fully expanded leaf attached to plants of five randomly selected transplants (n=5) in each treatment were used for this measurement. A custom- made method (Genty *et al*., 1989; Aliniaeifard *et al*., 2014; Aliniaeifard and van Meeteren, 2014) was used for the calculation of F_v/F_m. After reaching steady state fluorescence, during short measuring flash in darkness and during saturating light flash (by exposing to 3900µmol $m^{-2}s^{-1}$ saturating light pulse) F_0 and Fm were digitized and averaged, respectively. These two images were applied to obtain maximal variable fluorescence ($F_v = F_m - F_0$). F_v/F_m was calculated using expression $(F_m - F_0)/F_m$. The average value and standard deviation of F_v/F_m per image were calculated by Fluor Cam V.7.0 software.The same device (PAR-FluorPen FP 100 max, Photon System Instrument, PSI, Czech Republic) with the same method (20 minutes dark adaption) was used to measure OJIP-test whilethe last fully expanded intact leaf of randomly selected transplants was used to investigate biophysical and phenomenological parameters of Photo System II status (Strasser, 1995). The transient fluorescence measurement was induced by a saturating light of 3000 µmol $m^{-2}s^{-1}$. The OJIP transients were done according to the JIP test (Strasser *et al*., 2000). F_0, F_i, F_j, F_m, F_v, V_j, V_i, F_m/F_0, F_v/F_0, F_v/F_m, φP_0, φE_0, φD_0, ABS/RC, TR_0/RC, DI_0/RC, ET_0/RC, PI_{ABS} and ψ_0 were extracted using FluorPen software. More information on formulas are presented in Table 2.

Table 2 - Summary of OJIP-test formula using data extracted from OJIP chlorophyll fluorescence transient. Formulas

Formula abbreviation	Formula explanation
F_0	$F_0 = F_{50\mu s}$, fluorescence intensity at 50 µs
F_j	F_j= fluorescence intensity at J-step (at 2 ms)
F_i	F_i= fluorescence intensity at i-step (at 60 ms)
F_m	F_m= maximal fluorescence intensity
F_v	$F_v = F_m - F_0$ (maximal variable fluorescence)
V_j	$V_j = (F_j - F_0)/(F_m - F_0)$
V_i	$V_i = (F_i - F_0)/(F_m - F_0)$
F_m / F_0	
F_v / F_0	
F_v / F_m	
φP_0	$\varphi jP_0 = 1-(F_0/F_m) = F_v/F_m$
ψ_0	$\psi_0 = 1-V_j$
φE_0	$\varphi E_0 = [1-(F_0/F_m)] \times \psi_0$
φD_0	$\varphi D_0 = 1-\varphi P_0 - (F_0/F_m)$
PI_{ABS}	$PI_{ABS} = (RC/ABS)\times[\varphi P_0/(1-\varphi P_0)]\times[\psi_0/(1-\psi_0)]$
ABS / RC	$ABS/RC = M_0\times(1/Vj)\times(1/\varphi P_0)$
TR_0 / RC	$TR_0/RC = M_0\times(1/V_j)$
ET_0 / RC	$ET_0/RC = M_0\times(1/V_J)\times\psi_0$
DI_0 / RC	$DI_0/RC = (AB/RC)-(TR_0/RC)$
M_0	$M_0 = (TR_0/RC)-(ET_0/RC) = 4(F_{300}- F_0)/(F_m- F_0)$

ABS= absorption energy flux; CS= excited energy cross-section of leaf sample; DI= dissipation energy flux at the level of the antenna chlorophyll; ET= flux of electron from Q_A^- into the electron transport chain; φD_0= quantum yield of dissipation; φE_0= probability that an absorbed photon will move an electron into electron transport further than Q_A^-; φP_0= maximum quantum yield of primary photochemistry; PI_{ABS}= performance index; ψ_0= efficiency by which a trapped exaction, having triggered the reduction of Q_A to Q_A^-, can move an electron further than Q_A^- into the electron transport chain; RC= reaction center of PSII; RC/CS= fraction of active reaction centers per excited cross-section of leaf; TR, PSII; RC/CS= fraction of active reaction centers per excited cross-section of leaf; TR= excitation energy flux trapped by a RC and utilized for the reduction of Q_A to Q_A^-.

Pot stage (mature plants) measurements

Time to flowering and morphological characteristics. During pot stage, morphological traits such as number of flowers, flower diameter, number of leaves and number of days to flowering were measured and recorded.

Statistical analysis

Analysis of variance (ANOVA) was performed using SPSS (SPSS 15.0, SPSS Inc.) software and the means were compared with Tukey's test at $p \leq 0.05$.

3. Results

Transplant stage

Germination. Seeds grown under all LED lighting treatments performed better germination rate compared to SL. WBR, BR, WR and SL treatments had 96%, 94%, 96% and 87% germination, respectively (Table 3).

Morphological characteristics. Forty-five days after sowing seeds when all transplant had four fully expanded leaves, growth parameters were measured and analyzed (presented in Table 3). Seedlings grown under WBR and WR LED light exhibited significantly higher shoot fresh weight compared with control. Furthermore, shoot dry weight of plants grown under WBR and SL treatment had the highest and lowest average values, respectively. Average root fresh and dry weight values were maximum in WBR, however in the absence of blue LED, root biomass in WR-grown transplants was greatly reduced. All three LED lighting treatments had significantly greater canopy width than SL. Leaf area and leaf width of plants were significantly influenced by WBR light, though the SL treatment had the least average values. This prominence of WBR (with 33% blue LED ratio compared to 0% and 50%) was visible on leaf features and canopy width (Table 3).

Biochemical measurements

Plants grown under sunlight had the lowest chlorophyll a content while WBR and WR lighting treatments resulted in the highest content of chlorophyll a. LED light composed of blue and red had most profound effect on chlorophyll b synthesis. Control treatment and WR LED treatment had the lowest amount of chlorophyll b content. Additionally, identical proportion of white, red and blue light (WBR) led to the highest total chlorophyll (chl a+b) content among all the other treatments whereas SL had the lowest values. Carotenoid content was highly affected by WBR and WR LED lighting treatments while SL-treated plants showed the lowest carotenoid content (Table 3). Transplants grown under sunlight in greenhouse condition (SL) exhibited higher total soluble sugar content in comparison with all LED treatments (Table 3).

Chlorophyll fluorescence parameters

Measurements of chlorophyll fluorescence parameters were used to study the photosystem II activi-

Table 3 - Influence of light quality of white+blue+red (WBR), blue+red (BR), white+red (WR) and sunlight (SL) on germination, morphological and physiological characteristics of *Sinningia speciosa* transplants represented by means values ± standard deviation (n=5)

Parameters	Light quality			
	WBR	BR	WR	SL (as control)
Germination (%)	96 a	94 a	96 a	87 b
Shoot fresh weight (g)	1.122 ± 0.08 a	0.862 ± 0.05 b	1.084 ± 0.14 a	0.608 ± 0.12 c
Shoot dry weight (g)	0.065 ± 0.004 a	0.044 ± 0.003 bc	0.053 ± 0.01 ab	0.037 ± 0.012 c
Root fresh weight (g)	0.148 ± 0.008 a	0.088 ± 0.008 bc	0.080 ± 0.01 c	0.098 ± 0.008 b
Root dry weight (g)	0.0102 ± 0.0024 a	0.0084 ± 0.0011 ab	0.006 ± 0.0007 c	0.0078 ± 0.0013 bc
Canopy width (cm)	7.6 ± 0.3 a	7.9 ± 0.3 a	7.7 ± 0.02 a	6.1 ± 0.4 b
Leaf width (cm)	2.6 ± 0.1 a	2.4 ± 0.1 ab	2.5 ± 0.3 ab	2.2 ± 0.1 b
Leaf area (cm^2)	4.8 ± 0.3 a	3.6 ± 0.2 c	4.2 ± 0.5 b	3.2 ± 0.2 c
Chlorophyll a ($mg.g^{-1}$ FW)	0.122 ± 0.002 a	0.091 ± 0.026 ab	0.114 ± 0.031 a	0.077 ± 0.009 b
Chlorophyll b ($mg.g^{-1}$ FW)	0.089 ± 0.011 ab	0.099 ± 0.024 a	0.064 ± 0.022 b	0.064 ± 0.005
Chlorophyll a + b ($mg.g^{-1}$ FW)	0.212 ± 0.013 a	0.194 ± 0.027 ab	0.1946 ± 0.057 ab	0.141 ± 0.008 b
Carotenoid ($mg.g^{-1}$ FW)	2.840 ± 0.23 a	2.038 ± 0.47 ab	2.470 ± 0.77 a	1.580 ± 0.18 b
Total soluble sugar ($mg.g^{-1}$ FW)	49.42 ± 2.05 c	61.81 ± 1.98 b	47.05 ± 1.65 c	94.80 ± 0.65 a

Values followed by the same letter within a row do not significantly differ (by the tukey'stest, p≤0.05).

ty (Table 4). Based on this result, the fluorescence signal intensity of transplants grown under WR LED light, increased from F_0 to F_j and then to Fm, however, SL treatment showed the lowest values of extract and technical fluorescence parameters (F_0, F_i, F_j, F_m, F_v, V_i, F_m/F_0, F_v/F_0) as well as F_v/F_m. Transplants grown under WR LED lighting and control condition (SL) showed the highest and lowest values as for F_0, respectively. WBR and WR lighting treatments led to the significantly highest F_j value while it had the lowest significant value in SL treatment. All LED treatments (WBR, BR, WR) exhibited higher fluorescence yield at F_i, F_m, F_v compared to SL. The Highest V_j obtained in WBR and WR-grown plants and the lowest values were detected in BR-grown seedlings. The highest V_i value was from plants grown under WR lighting; however, SL treatment had the lowest value among the treatments. Plants exposed to BR LED light resulted in higher F_m/F_0value than other plants grown under different lighting conditions. F_v/F_0 was also decreased for all except BR-grown plants. The F_v/F_m ratio was higher in plants exposed to BR and WBR LED lights compared to SL which had the greatest decrease. Analyzed parameters for specific energy fluxes per reaction center (ABS/RC, TR_0/RC and DI_0/RC) increased in WR treated plants, in contrast BR light treatment had the greatest decrease among lighting treatments in DI_0/RC ratio while SL treatment had the highest increase of the same ratio. Also, SL and WBR showed the highest and lowest values of ET_0/RC, respectively. By analyzing the parameters that estimate quantum efficiencies or flux ratio (yields and efficiency of electron transport chain) the highest calculated values for φP_0, φE_0, PI_{ABS} and ψ_0 were obtained under BR treatment. In addition, plants grown under BR and WBR treatments had similarly the highest significant value in φP_0. Plants of WBR and BR treatments had the lowest values in φD_0 in compare with SL which had the highest values.

Pot stage (mature plants)

Morphological characteristics

Based on the results, there was a significant effect of LED lighting treatments on flower quantity where-

Table 4 - Chlorophyll fluorescence of transplants grown under white+blue+red (WBR), blue+red (BR), white+red (WR) and sunlight represented by means values ± standard deviation (n=5)

Parameters	Light quality			SL (as control)
	WBR	BR	WR	
F_0	9802 ± 1083 ab	8794 ± 663.5 bc	10517 ± 649 a	8031 ± 750 c
F_i	39013 ± 3447 a	36843 ± 2126 a	41150 ± 2688 a	29808 ± 3230 b
F_j	27919 ± 3109 a	23611 ± 2439 b	28439 ± 1890 a	20392 ± 2299 b
F_m	42752 ± 2254 a	39793 ± 2655 a	43443 ± 3162 a	32758 ± 3599 b
F_v	32949 ± 1755 a	30999 ± 2222 a	32925 ± 2530 a	24798 ± 2661 b
V_j	0.5508 ± 0.0628 a	0.4768 ± 0.0310 b	0.5448 ± 0.0236 a	0.4856 ± 0.018 ab
V_i	0.913 ± 0.022 ab	0.905 ± 0.011 bc	0.931 ± 0.013 a	0.886 ± 0.006 c
F_m/F_0	4.228 ± 0.171 b	4.535 ± 0.242 a	4.128 ± 0.069 b	3.944 ± 0.169 b
F_v/F_0	3.230 ± 0.171 b	3.535 ± 0.242 a	3.128 ± 0.069 b	2.944 ± 0.169 b
F_v/F_m	0.771 ± 0.021 a	0.778 ± 0.012 a	0.758 ± 0.004 ab	0.746 ± 0.011 b
φ_{P0}	0.771 ± 0.021 a	0.778 ± 0.012 a	0.758 ± 0.004 ab	0.746 ± 0.011 b
φ_{E0}	0.348 ± 0.059 b	0.408 ± 0.028 a	0.345 ± 0.019 b	0.387 ± 0.008 ab
φ_{D0}	0.229 ± 0.021 b	0.221 ± 0.012 b	0.242 ± 0.004 ab	0.254 ± 0.011 a
ABS/RC	3.449 ± 0.271 b	3.133 ± 0.170 c	3.771 ± 0.095 a	3.477 ± 0.038 b
TR_0/RC	2.654 ± 0.147 b	2.439 ± 0.128 c	2.857 ± 0.072 a	2.594 ± 0.01 bc
ET_0/RC	1.217 ± 0.070 b	1.273 ± 0.037 ab	1.301 ± 0.082 ab	1.337 ± 0.04 a
DI_0/RC	0.796 ± 0.127 ab	0.697 ± 0.060 b	0.914 ± 0.028 a	0.887 ± 0.048 a
PI_{ABS}	0.700 ± 0.149 c	1.163 ± 0.161 a	0.696 ± 0.072 c	0.895 ± 0.001 b
ψ_0	0.449 ± 0.063 c	0.523 ± 0.031 a	0.455 ± 0.024 bc	0.515 ± 0.018 ab

Values followed by the same letter within a row do not significantly differ (by the tukey's test, p≤0.05).

as the SL treatment resulted in the lowest number of flowers (presented in figure 2A). Largest flowers, in terms of flower diameter was detected in plants grown under WBR treatment, however WR treatment resulted in smallest dimeter of flowers (Fig. 2B). The results also indicated that treatments had significantly different number of days to flowering and plants grown under partial sunlight (SL) in greenhouse had longer time to flowering in pot stage in comparison with WBR, BR and WR-treated plants (Fig. 2D). The transplants grown under LED treatments had greatly higher number of leaves than SL at flowering stage (Fig. 2C).

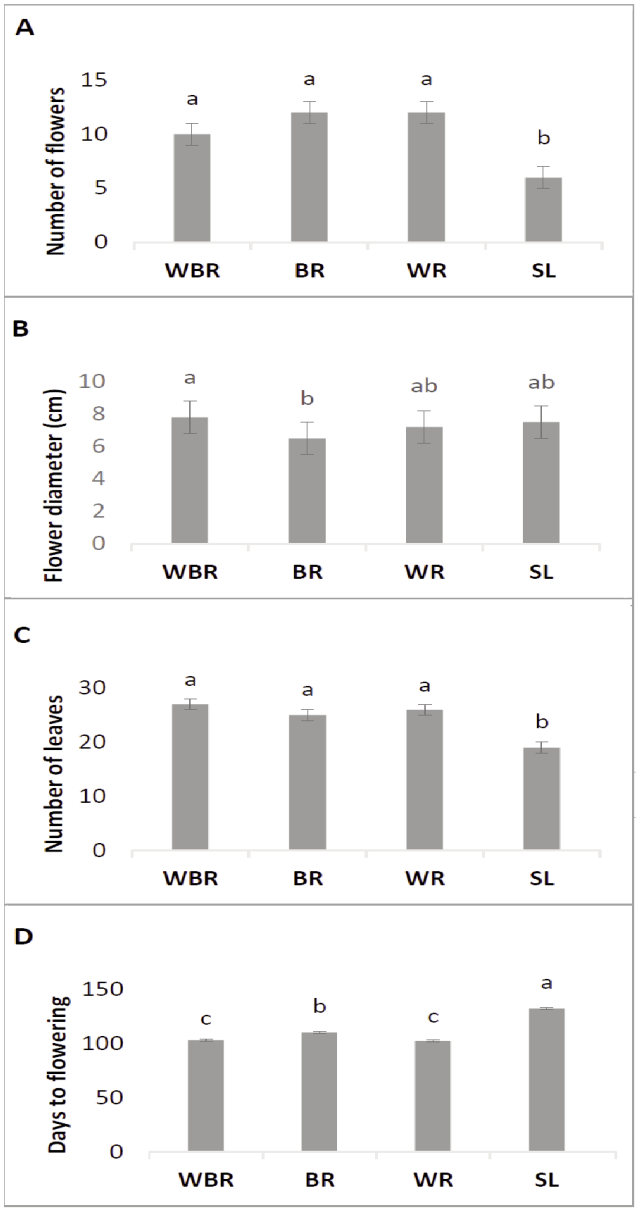

Fig. 2 - Morphological characteristics and time to reach flower of transplants grown under white+blue+red (WBR), blue+red (BR), white+red (WR) and sunlight in pot stage. The mean of values ± Standard deviation (n=5) is displayed. Values with the same letter in column do not significantly differ (by the tukey's test, p≤0.05). The explanations for the treatment abbreviations are provided in Table 1.

4. Discussion and Conclusions

In this experiment, significantly higher percentage of germination under LED light treatments, highlighted the effect of light quality on germination rate. It is known that orange/red and blue regions of light spectrum are most effective in germination process (Tozzi *et al*., 2005). Germination rate was satisfactory in absence of both blue and white LED lighting in WR and BR treatments, respectively. Overall, germination rate was highly affected under LED treatments compared to SL and it can be derived that presence of red light in all treatment resulted in a partially better germination in *Sinningia speciosa.*

Using different light spectra for tomato seedlings revealed that exposing seedlings to monochromatic red light showed higher shoot dry weight than 80% RB but small proportion of blue (95% RB) contributes getting more shoot dry weight (Gómez and Mitchell, 2015). Moreover, it was reported that lamps with substantial red but small blue waveband radiation energy, produced more weight yields in tomato compared to high blue and less red biased lamps (Warrington and Mitchell, 1976). Furthermore, among sodium lamp (1:1 RB), 100% R, 50% RB, 70% RB, 90% RB and white LEDs lighting treatments 90% RB led to significantly higher dry matter content (Wojciechowska, 2015). In another study with RW, RB, RBW and FL (fluorescent lamp) lighting treatments, WBR enhanced yield of lettuce plants including shoot FW, shoot DW, root FW, root DW and Leaf area (Lin *et al*., 2013). Our Results on fresh and dry weight and leaf features (leaf area and leaf width) were consistent with quoted reports and it can be concluded that a small blue proportion in combination with red and white spectrum will result in a higher biomass. Blue light is considered to be a substantial stimulator for leaf expansion which enhances leaf area and biomass production (Li *et al*., 2010; Cope ad Bugbee, 2013), on the other hand it has been proved that plant growth typically tends to decrease as the fraction of blue photons exceeds 5-10%, high levels of blue light in the spectrum results in inhibition of cell expansion, cell division, and leaf area expansion,

which ends up in less photon capture and diminished growth (Bugbee, 2016). Exposing to red light results in enhanced stem elongation (Hoenecke *et al.*, 1992), also absorbing high ratio of red light by plant photoreceptors can lead to production of a plant hormone called metatopolin (Steele, 2004), which can stimulate cell division as well as leaf expansion. Addition of white LED light may further increase plant growth, as white light might penetrate deeper in to the canopy and enhance photosynthesis compared to combination of red and blue light. Perhaps the combination of white, blue and red light perform a balanced spectral environment by providing a favorable amount of white light to plants (Lin *et al*., 2013).

White light is more capable of increasing chlorophyll than blue light, however some reports stated that blue light had significant effect on chlorophyll a synthesis (Wynne and Rhee, 1986; Rivkin, 1989; Aidar *et al*., 1994; Sanchez-Saavedra and Voltolina, 1994; Mercado *et al*., 2004; Hogewoning *et al*., 2010; Vadiveloo *et al*., 2015), also it was reported that red light plays an important role in chlorophyll content enhancement (Kubota *et al*., 1996). In addition, it has been reported combinational red light with blue and white light can increase carotenoid content accumulation (Lefsrud *et al*., 2008; Kopsell *et al*., 2014; Chen *et al*., 2016; Kopsell *et al*., 2016), however Lin *et al*. (2013) reported versus result, claiming that WBR LED has no effect on carotenoid content compared to RB LED and FL light. It can be concluded that white, red and blue light conjointly can enhance chlorophyll and carotenoid content but plant exact response to light quality varies with species and cultivars.

In present experiment, the maximum yield of primary photochemistry (φP_0) was in correlation with maximum quantum efficiency of photosystem II (F_v/F_m) which confirmed the enhancement of chlorophyll concentration under WBR treatment. We would suggest that transplants under WBR light treatment contained more chloroplast which maximized light capture for photosynthesis. Based on our results, we would suggest that the lowest photosynthetic rate in plants grown under SL is the result of low means of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content which was confirmed by the decline in F_v/F_m and φP_0 and increase in φD_0 and it can be concluded that increased φD_0 in SL treated transplants explains that due to highest amount of quantum yield of energy dissipation (φD_0), the major of natural light absorbed by the plant in high intensity was not used for the photochemical yield of electron transport chain and excess light dissipated as heat from the electron transport system (Aliniaeifard *et al*., 2018).

The higher total soluble sugar content of plants grown under SL compared to other plants grown under artificial LED lights, suggesting that under higher light intensity elevated level of soluble sugar helps transplants to avoid excessive light intensity and unlike LED treatments, they had less utility for photosynthesis (Ciereszko *et al*., 2001; Havaux and Kloppstech, 2001).

In this study, WR treatment showed the highest rate of increase in minimal fluorescence intensity (F_0) and this value decreased asproportion of blue light-but the opposite trend was observed as white proportion of light increased. This was also observed in F_i, F_j, F_m, F_v and $V_i$parameters. The maximum efficiency of photosystem II (F_v/F_m) increased in BR followed by WBR, however it was reduced in WR treatment (in the absence of blue LED). In an experiment using 32% BW and 40% BR lighting treatments on Phalaenopsis 'purple star 'showed higher amount in F_v/F_m in comparison with 0% BR (Ouzounis *et al*., 2014) which necessitates certain amount of blue light for proper photosynthesis (Hogewoning *et al*., 2010). F_v/F_m value ranges between 0.72-0.84 in many plants (Maxwell and Johnson, 2000), although the F_v/F_m of SL-grown transplants was in this range but it showed the least efficiency among other treatments, which is not surprising as this value changes with environmental conditions (Ouzounis *et al.*, 2014). Furthermore, it was shown that existenceof UV and yellow light in sunlight reduce photosynthesis efficiency (Takashi *et al*., 2010). In addition, *Sinningia speciosa* has no optimum photosynthetic activity under sunlight (Larson, 1992; Dole and Wilkins, 2005).

Transplants under highest blue proportion (BR) had the highest value in PI_{ABS} and φE_0 and the lowest value of DI_0/RC. The WR exposed transplants (which had no high ratio of blue light but high red ratio) showed the lowest PI_{ABS} and φE_0 and higher ABS/RC, TR_0/RC and DI_0/RC as the result. In an experiment investigating on photosynthetic and growth responses of purple variety of basil under white, blue and red LED lamps results shown that red light had the highest increase in ABS/RC, TR_0/RC and DI_0/RC and this amount was decreased under blue light (Hosseini *et al*., 2019). Inactivation of reaction centers and a decrease in active Q_A reducing centers occur as ABS/RC increases (Strasser and Stirbet, 1998). WR and WBR-grown transplants (with higher white and

red ratios) represented lower ET_0/RC which indicates that absorbed energy is briefly conveyed to the electron transport chain (Sarkar and Ray, 2016). This confirms that plants grown under BR light are more capable of transporting electrons from absorbed photons into electron transport chain and beyond $Q_A^{-1}\psi_0$ which could efficiently regulate energy level in the center of R reaction (Strasser *et al.*, 2004). SL-grown transplants showed highest soluble sugar content; however they had the second increase in PI_{ABS}, it is possible that in case of an environmental stress-such as high light intensity, in which excess energy beyond photosynthetic capacity is existing, led to production of ROS which results in oxidative damage to photosystem II (Pospíšil, 2016). In transplants grown under WBR and BR LED lighting, an increment in φP_0 value was observed, while there was no increase of the same value in transplants grown under sunlight. The results for φP_0 were in correlation with F_v/F_m which impacted chlorophyll and leaf area as explained in aforementioned chlorophyll measurements.

At flowering stage, transplants grown under LED lighting treatments resulted performed better ornamental criteria including number of flowers, flower diameter and number of leaves. Also it could be suggest that, those plants grown under LED lighting treatments could reach flowering stage sooner which will result in higher profit especially in commercial scale. Totally, in this experiment, this scenario was the case for plants grown under WBR treatment. Application of LED light in greenhouse in combination of red, blue and white wavelengths with a high photon efficiency are suitable for the production of horticultural plants (Kozai *et al*., 2015; Nicole *et al*., 2016). Our results are consistent with previous studies findings which indicate thatlighting source composed of red, blue and white light spectrum enhance morphological development of seedlings compared to monochromic light of each waveband (Brown *et al*., 1995; Gómez and Mitchell, 2015; Hogewoning *et al*., 2010; Ouzounis *et al*., 2014).

Desirable morphological and physiological characteristics in *Sinningia speciosa* transplants achieved under LED lighting treatments with identical proportion of white, blue and red led to enhanced morphological and physiological features at marketing stage including higher number of flowers, flower diameter, number of leaves as well as fewer days to flowering. Shorter time interval to flowering may help commercial growers to save time and costs of production while enhancing *Sinningia speciosa*plants quality in comparison with sunlight-grown transplants in conventional greenhouse condition. Moreover, it should be noted that using LEDs have higher expenses due to the cost of providing LEDs and growth chambers and also high consumption of electricity. Additional investigation is required to evaluate different ratios of spectral composition to optimize environmental condition for Gloxinia transplant production.

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Climatic and physiological parameters related to the progress and prediction of apple sunburn damage in a neotropical climate

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Abstract: Apple production in neotropical climate is affected by sunburn and the high interannual variability in meteorological conditions makes prediction and management of damage difficult. Non-destructive methods associated with physiological variables are keys to monitoring but their development is still incipient. In our study occurrence of sunburn, meteorological conditions and physiological parameters was monitored throughout four crop cycles. Fruit visual assessment and reflectance measures in field, as well as, pigments, proline and hydric potential in laboratory, were accomplished. The results show that the availability of water in the soil was more related to the evolution of sunburn than air temperature. Plant Senescence Reflectance Index (non-destructive predictor) discriminated between healthy and damaged fruits and fruit hydric potential and proline content were good indicators of sunburn, although such variables are determined when damage has already occurred. Our results suggest focusing future research on the water balance of the system and on the physiological indicators of osmotic stress as a way to predict damage.

1. Introduction

Fruit sunburn has been reported since 1870, and although several studies have touched upon the matter since the early 20th century (Racsko and Schrader, 2012), it is still a cause of significant economic loss in apple production (Reig *et al.*, 2019). Although some expressions of damage may be easily perceived in the field, on occasions the symptoms of sunscald are imperceptible and only appear after months of cold storage (usually after three months), which makes the damage difficult to control (Yuri *et al.*, 2000). Symptoms appear as brown stains on the fruits'

exterior, being Granny Smith the most susceptible reported cultivar (Felicetti and Schrader 2008; Hernandez *et al*., 2014).

There is consensus that sunburn is related to the combination of high temperature and irradiance (UV-B range is thought to be essential) during the fruit growth period (Yuri *et al.*, 2000, 2010; Racsko and Schrader, 2012; Torres *et al*., 2013; Darbyshire *et al*., 2015; Torres *et al*., 2016 a, b), however, there is a limited understanding of the physiological aspects of the changes in the fruit's internal quality (Racsko and Schrader, 2012) and generation of sun-related physiological disorders in fruit. This problem requires further research into the environmental and physiological processes that occur prior to and during sun injury development and more importantly, biochemical changes that may contribute to resistance to environmental conditions that cause sun-related disorders in fruit (Morales-Quintana *et al*., 2020).

Stress adaptation mechanisms of plants, such as chlorophyll reduction (Ballester *et al*., 2017), dissipation of excitation energy, increase of solutes of low molecular weight (Wen and Moriguchi, 2015) and changes in pigmentation (Merzlyak *et al*., 2003) have been previously studied in relation to sunburn in apple fruit. Based on these results, in the last decade, work has been done on the development of non-destructive methods to predict and detect sun damage based on the composition and location of skin pigments as well as the optical properties of the underlying fruit tissue (Solovchenko *et al*., 2010; Torres *et al*., 2016 a, b). In this direction, sunburn has been related to: fruit reflectance values in the visible and near-infrared (NIR) spectra (Solovchenko *et al.*, 2010; Torres *et al*., 2016 a), crop water stress index and chlorophyll fluorescence (Torres *et al*., 2013, 2016 b).

About temperature effect, it has been reported that increases in fruit temperature above a certain limit may cause enzymes denaturation and protein coagulation, leading to tissue damage (Yuri *et al*., 2010). Studies performed in cv. Fuji fruits showed a highly susceptible caused by excessive heat and did not sustain damage when exposed to UV radiation only (Yuri *et al*., 2000). Air temperatures of 38-42°C increases the heat-shock proteins induction (Woolf and Ferguson, 2000) and sunburn symptomatology appear with fruit temperature of 46°C and higher (Racsko and Schrader, 2012).

The water status of the plant and fruit has also been related to sunburn, although fewer studies have focused attention on this aspect. Recent work in Chile analyses the association of acclimation events with fruit water relations and osmoregulation occurring in sun-exposed fruit tissue (Torres *et al.*, 2013). Studies in Japan and South Africa discuss the effect of foliar ABA on antioxidant levels and the incidence of sunburn with variable results (Mupambi *et al*., 2018). Antioxidant system plays a crucial part in the elimination of free radicals under stress conditions (Chen and Murata, 2002). Compatible solutes such as proline, betaine and polyols are accumulated in response to abiotic stress (Suzuki, 2015). These solutes affect the osmotic balance and the membrane stability and have been proven to maintain turgor pressure, cellular volume and electrolyte concentration (Roberts, 2005). Proline is known to be a stabilizer of sub-cellular structures (Kautz *et al*., 2015) and although many studies have established a connection between proline and antioxidant activity in apple plant leaves and xylem under abiotic stress (Šircelj *et al*., 2005; Nemeskéri *et al*., 2015; Afonso *et al*., 2017) no relationship between proline and sunburn has yet been reported.

Most of the existing research has been carried out in latitudes similar to that of the present study but in more arid climates such as, Chile, Australia, and South Africa (southern hemisphere) or Spain, Turkey, and Washington State (north hemisphere), however, few studies have addressed sunburn in humid growth-season conditions like in Eastern New York State (Reig *et al*., 2019). The region where the study was conducted, defined as neo-tropical (Bernardi *et al*., 2016), has been considered restrictive for apple quality in relation to sunburn aspects (FAO-MGAP, 2013) due to the occurrence of high temperatures during the fruit growth period. Changes in El Niño evolution after 1976 may have played a role in altering the relationship between temperature extreme events in Uruguay and the atmospheric circulation (Renom *et al.*, 2011). The average maximum temperatures of the summer period show a high iner-annual variability (71-86%) and lower variability in the medium (10 years) or long-term (>30 years) components, 23% and 6% respectively (Tiscornia *et al*., 2016), so it is expected that the climate in the region will continue to be favorable to the occurrence of burning. The aims of this work were to study apple sunburn progress and its relation to meteorological variables in a neo-tropical climate, and to establish correlations between fruit physiological parameters and reflectance index.

2. Materials and Methods

Plant material

The experiment was conducted during the 2012/2013 to 2015/2016 crop cycles (hereinafter, cycles 1 to 4), on a Granny Smith/M7 plantation established in 2003. The crop is located in Uruguay (southeastern of South America) with the coordinates of 34°38'18'' S and 56°40'06'' W and 45 meters above sea level.The climate of this regional ecotone is classified by Bernardi *et al*. (2016) as neo-tropical. Crop had planting distance of 4x1.5 m, rows arranged from N to S and trained in central leader system. The soil types are mainly Argiudolls and Hapluderts and a drip irrigation system with a maximum daily watering capacity of 4.5 mm is installed.

Three fruits per tree from ten trees per row, in a total of five rows were selected between 40 and 50 days after full bloom (DAFB) in the four evaluated cycles. Trees and rows were randomly marked, and 150 fruits exposed to radiation were classified by visual assessment of different external initial conditions: A) 50 fruits with no visible sunburn (HF=healthy fruits); B) 50 fruits with red color (RF=red fruits); C) 50 fruits with an early degree of sunburn (SBF=sunburn fruits) [sunburn browning, according to the classification of Racsko and Schrader (2012)] as indicated in figure 1. The flowering dates for cycles 1 to 4 were, September 27 (cycle 1), October 28, 3 and 14, to cycles 2, 3 and 4 respectively.

The exposed side of each fruit was defined as the one directly exposed to sunlight, facing the space between rows, and the internal side as the one facing the trunk, with no direct exposition to solar radiation.

Field tests

The sunburn progress in each marked fruit was assessed by observation. Its frequency varied between 1 week and 1 month, with weekly observations predominating. In each observation, fruits were reclassified according to the categories mentioned above (HF, RF, SBF). Reflectance measurements of the exposed side of ten fruits of each condition were recorded with an ILT 950 spectroradiometer (International Light Technologies, USA) at 91, 99 and 154 DAFB in cycle 4. Percent reflectance was calculated based on a dark spectrum and a reference spectrum from a white reference standard. The content of Chlorophyll (CHL), Anthocyanins (ANT), Carotenoids (CAR), Flavonoids (FLA) and Senescence indexes were calculated based on the reflectance measurements. Chlorophyll was calculated according to the following indexes: CHL1, CHL2 (Mullan, 2013) RARSa, RARSb, PSSRa, MSR, CL1, CL2 (Solovchenko *et al*., 2010) and Anthocyanins, according to the Anthocyanin Reflectance Index (ARI) (Solovchenko *et al*., 2010). Carotenoids were calculated according to the following indexes: RARSc (Mullan, 2013), CRI1 and CRI2 (Solovchenko *et al*., 2010) and Flavonoids according to the Flavonoid Reflectance Index (FRI) (Solovchenko *et al*., 2010). The Normalized Phaeophytization Index (NPQI), the Pigment Simple Ratio (PSR), the Normalized Difference Pigment Index (NDPI), the Structural Independent Pigment Index (SIPI) (Solovchenko *et al.,* 2010) and the Plant Senescence Reflectance Indexes (PSRI480 PSRI500) (Mullan, 2013) were also calculated (Table 1). An automated meteorology station located 1900 m from the crop recorded the maximum temperature (Tmax) (°C) and rainfall (RF) (mm) variables in the fruit's growth period. The soil water balance (SWB) for each growth cycle was calculated. Plot characteristics and local and regional meteorology stations were used. Local variables used were irrigation (mm), daily rainfall (mm), root deep (m), phenological stages (days) and soil texture. The ETo (reference evapotranspiration) (mm) was recorded at the meteorology station of INIA Las Brujas using Penman-Monteith (Allen *et al.,* 1998). Crop coefficient (Kc) was adjusted to reflect the wetting frequency of soil surface and local climatic conditions according to Allen *et al.* (2006):

Fig. 1 - Examples of fruit categories. RF=red fruits, HF=healthy fruits, SBF=sunburn fruits.

$$K_{cini} = K_{cini\,(*)} + \frac{(I-10)}{(40-10)} [k_{cini\,(**)} - K_{cini\,(*)}]$$

$$K_{cmid} = K_{cmid}\,(Tab) + [0.04\,(u_2 - 2) - 0.004\,(RH_{min} - 45)]\,(\frac{h}{3})^{0.3}$$

Where:

$K_{cini(*)}$: value for Kc ini from figure 29 in Allen *et al*. (2006).

$K_{cini(**)}$: value for Kc ini from figure 30 in Allen *et al*.

(2006).
I: average infiltration depth (mm).
$K_{c\ mid}$ (Tab): value for Kc mid taken from Table 12 in Allen *et al*. (2006) apples, cherries and pears crops with active ground cover without frosts.
u_2: mean value for daily wind speed at 2 m height over grass during the mid-season growth stage (m s^{-1}), for 1 m s^{-1}<u_2<6 m s^{-1}.
RHmin: mean value for daily minimum relative humidity during the mid-season growth stage (%), for 20%<RHmin< 80%.
h: mean plant height during the mid-season stage (m) for 0.1 m < h < 10 m.

Laboratory

Five fruits of each damage category were sampled on the same dates on which the reflectance measurements were carried out (91, 99 and 154 DAFB) and in addition to the 112, 142 and 170 DAFB of cycle 4 for laboratory evaluations. Hydric potential of the fruits' tissue (ΨF) was determinate with Wp4c dewpoint potentiometer (Decagon Devices, USA) on the exposed side (ΨFE) and the internal side (ΨFI). Each measurement was made on 3 cm^2 of epidermis with 1 mm of sub-epidermal tissue. To determine CHL, CAR and Proline content (PRO), 0.5 g of epidermis without sub-epidermal tissue was removed. The sample was macerated with 0.5 ml of a methanol-chloroform-water compound (MCW, 12:5:1), producing two phases. In phase 1, pigments CHLa, CHLb and CAR were determined, recording absorbances at 665.6, 647.6 and 480 nm, respectively (Wellburn, 1994). In phase 2, after reacting to a one-hour immersion at 90°C with acid ninhydrin and the addition of toluene, PRO was determined with spectrophotometry, recording absorbance at 515 nm (Troll and Lindsley, 1955; Charest and Phan, 1990). To determine ANT, 0.2 g tissue was incubated in methanol-acid for 48 h, recording absorbance values at 530 and 657 nm (Wellburn, 1994).

Statistical analysis

The contrastive analysis of the three fruit conditions regarding pigment quantification and assessment, PRO, ΨF and senescence indexes were done with non-parametric methods, using the Kruskal-Wallis test to compare medians and the Kruskal Nemenyi post-hoc test for the multiple comparisons of pairs, with the R statistical software. Differences were assessed at p≤0.05. Pearson's correlation coefficient was determined for the proportion of sun-damaged fruits and the climatic variables, maximum temperature (Tmax) and soil water balance (SWB). The same analysis was performed for the pigment content tested in the laboratory and those estimated by spectroradiometry in the field, as well as for PRO and ΨF. Best fit regressions for variables with higher correlation coefficients were estimated.

Table 1 - Indexes calculated by reflectance in the field

Name	Index	Index calculation	Parameter	Source
Reflectance Ratio	CHL1	R_{750}/R_{550}	Chlorophyll	Braun and Payne, 2013
	CHL2	R_{750}/R_{700}		Braun and Payne, 2013
Ratio Analysis of Reflectance Spectrum (Chla)	RARSa	R_{675}/R_{700}	Chlorophyll a	Braun and Payne, 2013
Ratio Analysis of Reflectance Spectrum (Chlb)	RARSb	$R_{675}/(R_{650}*R_{700})$	Chlorophyll b	Braun and Payne, 2013
Ratio Analysis of Reflectance Spectrum (Carotenoids)	RARSc	R_{760}/R_{500}	Carotenoids	Braun and Payne, 2013
Pigment-Specific Simple Ratio	PSSRa	R_{800}/R_{675}	Chlorophyll a	Braun and Payne, 2013
Normalized Phaeophytization Index	NPQI	$(R_{415}-R_{435})/(R_{415}+R_{435})$	Chlorophyll degradation	Braun and Payne, 2013
Modified Spectral Ratio	MSR	$(R_{750}-R_{445})/(R_{705}-R_{445})$	Chlorophyll concentration	Braun and Payne, 2013
Pigment Simple Ratio	PSR	R_{430}/R_{680}	Carotenoid-Chlorophyll ratio	Braun and Payne, 2013
Normalized Difference of Pigment Ratio	NDPI	$(R_{680}-R_{430})/(R_{680}+R_{430})$	Carotenoid-Chlorophyll ratio	Braun and Payne, 2013
Structural Independent Pigment Index	SIPI	$(R_{800}-R_{435})/(R_{415}+R_{435})$	Carotenoid-Chlorophyll ratio	Braun and Payne, 2013
Chlorophyll Index	CL1	$(R_{700}^{-1}-R_{800}^{-1})* R_{800}$	Chlorophyll	(Solovchenko *et al*., 2010)
	CL2	$(R_{640}^{-1}-R_{800}^{-1})*R_{800}$	Chlorophyll	(Solovchenko *et al*., 2010)
Anthocyanin Reflectance Index	ARI	$(R_{550}^{-1}-R_{700}^{-1})* R_{800}$	Anthocyanin	(Solovchenko *et al*., 2010)
Plant Senescence Reflectance Index	$PSRI_{480}$	PSRI480=(R678-R480)* R-1800	Carotenoid-Chlorophyll ratio	(Solovchenko *et al*., 2010)
	$PSRI_{500}$	PSRI500=(R678-R500)* R-1800	Carotenoid-Chlorophyll ratio	(Solovchenko *et al*., 2010)
Flavonoid Reflectance Index	FRI	$(R_{410}^{-1}-R_{460}^{-1})* R_{800}$	Flavonoids	(Solovchenko *et al*., 2010)
Carotenoid Reflectance Index	CRI_1	$(R_{520}^{-1}-R_{700}^{-1})* R_{800}$	Carotenoids	(Solovchenko *et al*., 2010)
	CRI_2	$(R_{520}^{-1}-R_{550}^{-1})* R_{800}$		(Solovchenko *et al*., 2010)

3. Results

Sunburn progress in the four crop cycles showed a high inter annual variability, with differences in the incidence values (% of damaged fruits) and the moment when the maximum occurs. Maximum values of 70% and 62% of sunburnt fruits were recorded in late December and early January for cycles 2 and 3 respectively (approximately 12 and 14 weeks after full bloom). For cycles 1 and 4, maximum values under 45% were recorded between mid-February and early March, at 19 and 22 weeks after full bloom (Fig. 2).

From the analysis of the meteorological records of the four cycles, it can be highlighted that, in cycle 2, 11 days were recorded with maximum temperatures above 35°C, starting at 68 DAFB and reaching a maximum of 38.9°C at 77 DAFB. Cycles 1 and 3 respectively recorded 1 and 0 days with maximum temperatures above 35°C. In cycle 4, 5 days had a maximum higher than 35°C with only 1 peak above 38°C (Fig. 3).

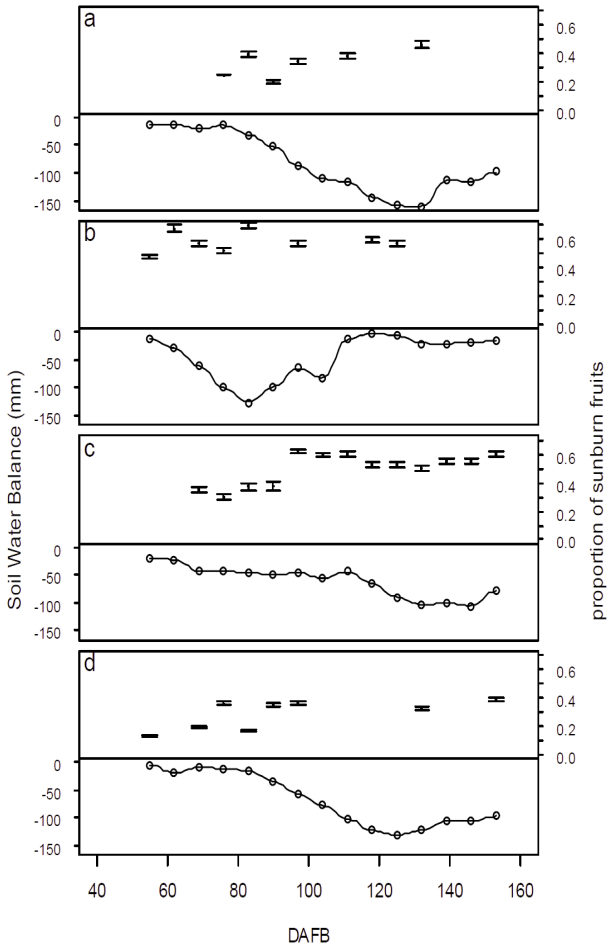

Fig. 2 - Proportion of sunburn fruits and soil water balance (SWB) by number of days after full bloom (DAFB) for four production cycles. a, cycle 1; b, cycle 2; c, cycle 3; d, cycle 4. The error bars represent standard deviation.

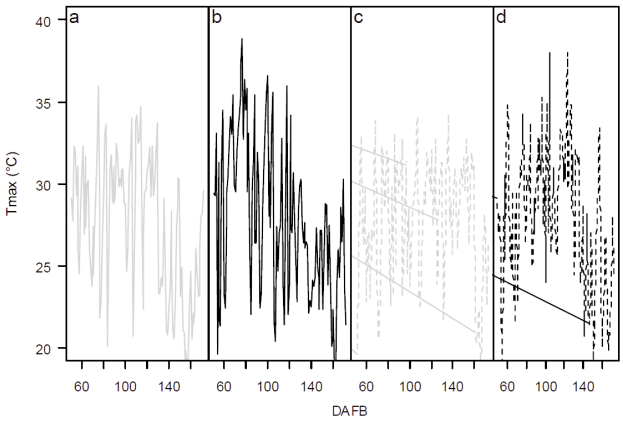

Fig. 3 - Air maximum temperature (Tmax) by days after full bloom (DAFB) for four production cycles. a. cycle 1; b. cycle 2; c. cycle 3; d. cycle 4.

Lowest values of SWB during the first 80 DAFB (about 11 weeks), in the period when damage appears, were recorded in cycles 2 and 3 showing minimum values of -123 and -46 mm respectively. On the other hand, in cycles 1 and 4, SWB decreases from week 12 (Fig. 2). Pearson's correlation coefficient between the proportion of sun-damaged fruits and the soil water balance (SWB) was -0.41, with variations between -0.37 and -0.7 depending on the cycle. The correlation with maximum temperature was 0.28, with a variation of between -0.1 and 0.51 for the different cycles (Fig. 4).

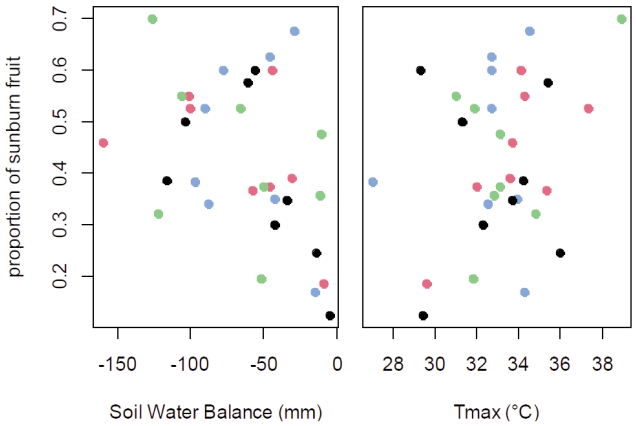

Fig. 4 - Correlations between proportion of sunburn fruits and climatic variables. a. Soil Water Balance (SWB); b. Air maximum temperature (Tmax). Colors represent each cycle: black for cycle 1, red for cycle 2, green for cycle 3,

Variables such as CHL, CAR, ANT, PRO, ΨF and Stress Index, showed significant differences for at least one of the three kinds of fruit (HF, SBF, RF) when measured in the laboratory and/or when estimated in the field, except for ΨFI and CHLb content measured by spectrophotometry (RARSb). ANT concentration, measured in laboratory, and MSR (field

CHL estimate), differed significantly between the three types of fruit. ΨFE was significantly different between SBF and the other two conditions (RF and HF). Remaining variables differed significantly between HF and RF or SBF, but not between RF and SBF. Higher PRO concentrations were measured in RF and SBF than HF fruits (Fig. 5, Table 2).

Pearson correlation coefficients calculated for the different variables assessed reached a maximum of -0.75 between ΨFE and PRO. CHL concentration measured in the laboratory had negative correlations of 0.42 with ΨF and 0.57 with PRO (Fig. 6). The relationship between ΨFE and PRO, CHLa and ΨFE, NDPI and PSRI480, 860900 and CHLa, and NDPI and CHLa have the best fit with a 2nd degree polynomial regression (Fig. 7) and a maximum r^2 of 0.60.

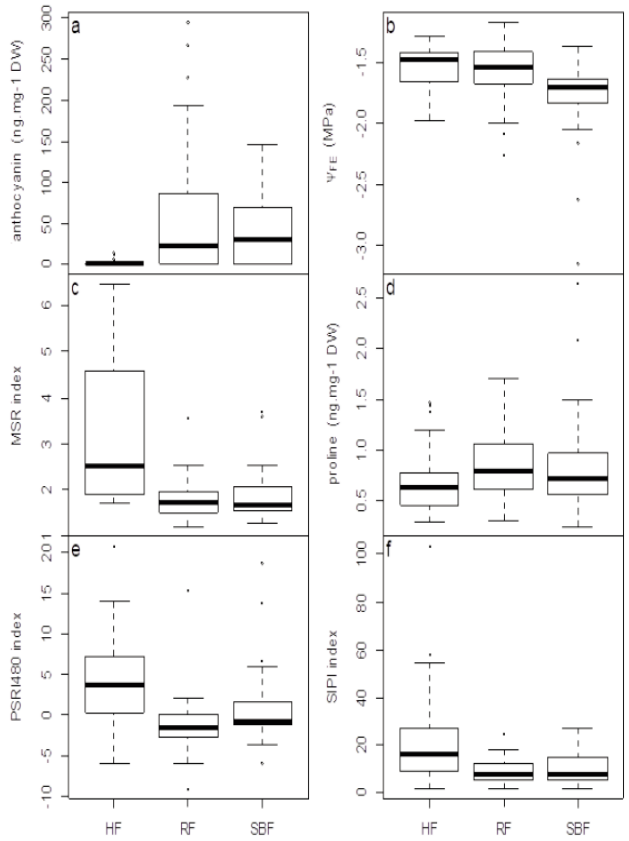

Fig. 5 - Boxplot of variables with greater discrimination capacity for the three types of fruit (HF= healthy fruit; SBF= sunburnt fruit; RF= red fruit). a) anthocyanin concentration; b) hydric potential of the exposed side of the fruit; c) MSR (modified spectral ratio, chlorophyll concentration estimate by reflectance); d) proline concentration; e) PSRI480 (Plant Senescence Reflectance Index); f. SIPI (Structural Independent Pigment Index).

Table 2 - Median of anthocyanins (ANT), Chlorophyll (CHL), Carotenoids (CAR) and proline (PRO) concentration, hydric potential of the exposed side of the fruit (ΨFE), Reflectance Ratio (CHL2), Chlorophyll concentration estimate by reflectance (MSR, Modified Spectral Ratio), Structural Independent Pigment Index (SIPI) and Plant Senescence Reflectance Index (PSRI480), for the three types of fruit (healthy fruit, sunburnt fruit, and red fruit)

	Sunburnt fruit	Red fruit	Healthy fruit
ANT (ng.mg^{-1} DW)	29.871 b	23.438 a	0 c
CHL (µg.mg^{-1} DW)	0.034 b	0.041 ab	0.045 a
CAR (µg.mg^{-1} DW)	0.009 b	0.011 ab	0.012 a
PRO (ng.mg^{-1} DW)	0.724 a	0.788 a	0.64 b
Ψ_{FE} (MPa)	-1.705 a	-1.54 b	-1.475 b
CHL2	1.808 b	1.785 b	2.658 a
MSR	1.660 a	1.716 b	2.504 c
SIPI	8.239 b	8.052 b	16.012 a
PSRI480	-0.843 b	-1.534 b	3.65 a

Different letters in rows indicate significant difference between types of fruit

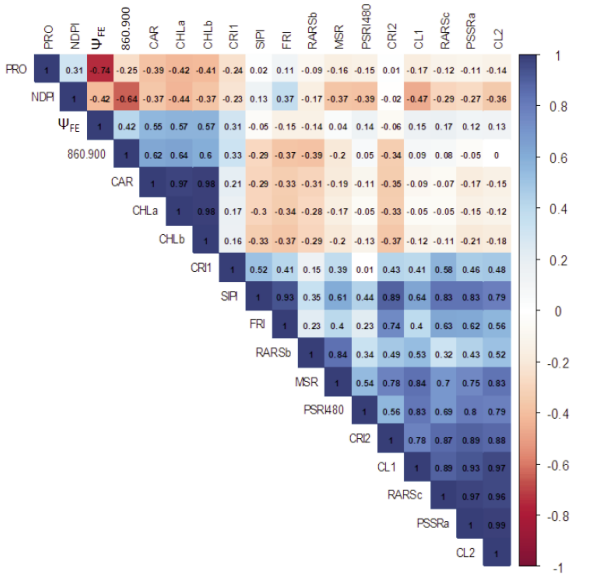

Fig. 6 - Correlations between variables with greater discrimination capacity for the different fruit conditions [Proline (PRO), Carotenoids (CAR), Chlorophylls (CHL)] measured by spectrophotometry in the laboratory, NDPI, 860-900, CRI1, SIPI, FRI, RARSb, MSR, PSRI480, CRI2, CL1, RARSc, PSSRa and CHL2, calculated based on reflectance measurements in the field.

4. Discussion and Conclusions

Sun damage incidence values reported in our trial reached a maximum of 70%. This was higher than reported by Racsko and Schrader (2012) in warmer climates of Australia, South Africa, Spain, Turkey and

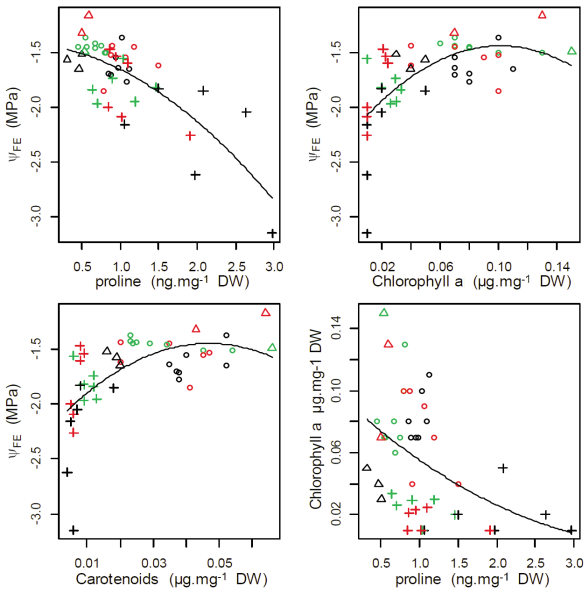

Fig. 7 - Regressions between laboratory variables. Colors represent condition of fruit: green for healthy fruit (HF), red for red fruit (RF), and black for sunburnt fruit (SBF). Symbols represent days after full bloom (DAFB): ○ for 91; Δ for 99; + for 154.

Chile, and higher than the 40% reported by Yuri and Bailey (2015) for Chilean crops. Similar sunburn values were reported during a heat wave in southeast Australia in 2009 (Darbyshire *et al*., 2015). All cycles differed, both in how the damage progressed and at the time when the maximum damage occurred. These differences can be clarified by analysing the meteorological variables, which, as expected, were also highly variable (Tiscornia *et al*., 2016). The correlation of sun damage with SWB was moderate to strong (0.37 to 0.7 depending on the cycle) and higher than that presented with maximum temperatures (Fig. 4). In cycle 2, 11 days with maximum temperatures above 35°C along with the SWB progress, could explain the occurrence of sunburn. However, in cycle 3, only the early decrease in SWB seems to be the explanatory variable of a high damage incidence (Figs. 2 and 3).

In four cycles studied, sunburn reached maximum values at 19, 12, 14 and 22 weeks after full bloom (Fig. 2). In all cases, minimum fruit diameter reported as necessary to absorb enough solar radiation to increase the temperature and result in damage (45 mm 7 weeks) (Racsko and Schrader, 2012) had reached. Fruit surface temperatures between 46 and 49°C, depending on the cultivar, cause browning, while temperatures over 52°C cause necrosis (Schrader *et al.*, 2001). This is in accordance with fruit temperatures recorded by Darbyshire *et al*. (2015) for Australia, as well as other reports suggesting that the temperature of the exposed side of the fruit may be 12 to 15°C higher than air temperature (Woolf and Ferguson, 2000). Fruit temperatures over 52°C were recorded in our trials with thermal camera. On days with average air temperatures above 38°C (in cycle 2) were reached fruit temperatures up to 62°C (data not shown).

Variations in ΨF and pigment concentration (laboratory measurements or estimates with reflectance in the field), measured in three types of fruit (HF, RF, SBF) (Fig. 5), have been reported in previous works by Solovchenko *et al*. (2010) and Torres *et al*. (2016 a, b). Symptoms of sun damage were associated with increase in CAR and reduction in CHL and ΨF, as reported by Felicetti and Schrader (2009), Torres *et al*. (2013, 2016 b) and Yuri *et al*. (2000). CHLb estimated by reflectance did not decrease.

ΨFE was the only discriminating variable in SBF vs HF and RF (Fig. 5), in accordance with Torres *et al*. (2013). Increase of 860-900 nm reflectance, reported as a consequence of structural differences between damaged and undamaged tissue (Torres *et al*., 2016 a), could not be confirmed by spectroradiometry in our work. A 0.42 correlation between ΨFE and reflectance values in the 860-900 nm range were obtained (Fig. 6).

Although it has been widely reported that the content of PRO in vegetables tissues is an indicator of stress (Suzuki, 2015), correlations between PRO, CHL and ΨF (Fig. 6) in apples have not been reported. Research on apple trees under hydric stress conducted by Šircelj *et al.* (2005) does not report consistent patterns of change of individual free amino acids. These authors, however, mention the lack of agreement between those results and their own previous studies, as well as those by Chandel and Chauhan in 1991, which report significant increases in Pro foliar, Glu, Orn, Arg and total free amino acids under stress, evidencing active osmoregulation (Šircelj *et al.*, 1999). Our results showed an increase in PRO content in sunburn fruits. More studies should be carried out to evaluate the possibility of using this amino acid as an early indicator of sun damage. Total amino acid content and its variations should be analyzed to elucidate between de novo PRO synthesis and simple proteolysis (Šircelj *et al*., 2005; Arias-Sibillotte *et al*., 2019).

In accordance with the results of Felicetti and

Schrader (2009), the correlations found between CHL and CAR concentrations were significant and negative, whereas the correlation between the values of pigments measured in the laboratory and those estimated by reflectance reached a maximum of 0.25 (Fig. 6). Plant senescence index PSRI480 was the spectroradiometry indicator with the best capacity to discriminate between fruit types (Fig. 5). Correlation between PSRI480 and the best-performing laboratory indicators (PRO y ΨF) was 0.15 (Fig. 6). Pigment concentration expressed on the basis of surface area were lower than range cited by Solovchenko *et al*. (2010) in a 1/50 ratio, but similar to those recorded by Yuri *et al*. (2010), expressed as dry weight concentration. This difference could explain the weak correlation between the laboratory tests of pigments and field estimates in our trials.

High inter-annual variability of sunburn in apple fruit was observed in terms of magnitude and moment of occurrence. The inter-annual variability of rainfall combined with insufficient irrigation to meet crop water demand was more associated with sunburn during the period studied than the maximum temperature. Regarding non-destructive prediction, the PSRI480 senescence reflectance index was the best discriminator between undamaged and damaged fruits, however, like other recent studies, we were unable to devise a method of non-destructive prediction that could be used for commercial production. ΨFE is the main variable that discriminates the degree of damage to the fruits, so it could be the consequence of the effects of high irradiance and temperature. The association between low hydric potential and PRO contents in sunburn fruits suggests the possibility of using this amino acid as an early indicator of this damage in apples; however, more information is necessary to establish a cause and effect relationship.

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AHS
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Variations in tree and fruit characteristics revealed potential dwarfing genotypes within Iran's pomegranate germplasm

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Abstract: This study aimed to explore diversity in dwarfing tendencies, determine the correlation of measured traits with dwarfing, and identify and select promising dwarf candidates as potential scions or rootstock cultivars. Growth habit, vegetative attributes, fruit physicochemical characteristics,and leaf stomatal densityof 19 Iranian pomegranate cultivars, which have been collected across the country and established in the Yazd pomegranate germplasm, were assessed. Results showed that the cultivars differed in almost all measuredtraits. The tree height and canopy width, current year's shoot, and internode length were within the range of 1.97-4.6 m, 1.53-4 m, 15-41.5 cm 1.96-3.39 cm, respectively. Moreover, a positive correlation was observed between tree height and internode length (r= 0.55), whereas a negative correlation was obtained between stomatal density and tree height (r= -0.44). Based on characteristics measured for the vegetative growth, 'Malas No. 1 Saravan' and 'Torosh Nar Riz Zirab' proved dwarfing habit. 'Rabab Poost Ghermez Neyriz',a commercial cultivar, showed semi-dwarfing growth and 'Khajei Ghasrodasht Fars', 'Shahsavar Seydan Marvdasht', 'Bihaste Ravar', 'Bihaste Sangan Khash', 'Torosh Goli Naz Behshahr' and 'Anar Siah' resulted in vigorous trees. This preliminary study found promising dwarf and semi-dwarf genotypes at Iran's pomegranate germplasm.

1. Introduction

According to historical documents, pomegranates originated in central Asia, especially in parts of Iran, and believed to have spread to nearby areas due to traveling and incursion (Harlan, 1975; Levin, 1994; Verma *et al.*, 2010). The main Iranian collection of pomegranate in Yazd contains 762 accessions, including wild, semi-wild, and commercial types (Behzadi Shahrbabaki, 1998; Zamani *et al.*, 2007). These diverse and valuable

genetic resources would benefit from a further genetic improvement to develop dwarfing scion and rootstock genotypes to establish modern high-density pomegranate orchards. Ingels *et al.* (2002) suggested that the term 'dwarf tree' applies to a tree that appears smaller than usual owing to selection of dwarf genotypes, specific training or pruning methods, or grafting on dwarfing rootstocks. Besides, in classifying trees according to size, dwarf trees are approximately 2.5 m or less when they mature (Castle, 1992). Dwarf trees have many benefits compared to larger vigorous ones, such as being able to be spaced closer together without suffering from excessive crowding or the need for frequent, severe pruning. Moreover, dwarf trees allow for ease of pruning, pest control, fruit thinning, spraying, harvesting, and increased production of high-grade fruit, higher fruit quality and decreased production costs (Tukey, 1964). The advantages of dwarf and semi-dwarf genotype trees have been demonstrated in the fruit industry resulting in the widespread use of dwarfing rootstocks in tree crops such as apple (Looney and Lane, 1983), cashew (Moura, 2001), and peach (DeJong *et al.*, 2005). Lately, breeding efforts have resulted in the selection of dwarfing rootstock or scion cultivars in almost all temperate and tropical fruit tree crops (Busov *et al.*, 2003). The primary identification of dwarf genotypes should be based on field evaluations of fruit tree cultivars and genotypes. Genetic dwarfism often manifests itself in distinctive morphological characteristics, easily identified, and often appears to a casual observer (Castle, 1992). Analysis of morphological diversity as tree height, canopy shape, internode, and branching pattern is a useful method for detecting dwarfing phenotypes in a population of many genotypes. Although some pomegranate cultivars, such as Nana, are considered dwarfing (Terakami *et al.*, 2007), these are ornamentals, and there are no published reports on new dwarfing pomegranates. The objectives of this study were: 1) evaluate tree growth habits, vegetative and fruit characteristics of 19 Iranian pomegranate cultivars, 2) to examine diversity in dwarfing potential, 3) determine inter-correlations among measured traits with dwarfism, and 4) identify promising candidates with dwarfing potential as pomegranate scions and rootstocks.

2. Materials and Methods

Plant materials

The experiment was conducted in 2015, at the Agriculture and Natural Resources Research Centre

Table 1 - Name, origin, fruit and tree characteristics of pomegranate cultivars used in this study

Cultivars	Provinces	Cities	Skin color	Aril color	Taste	Uses
Anar Siah	Esfahan	Esfahan	Black	Dark red	Sweet	Medicinal
Bihaste Ravar	Kerman	Ravar	Yellow-pink	White	Sweet	Local
Bihaste Sangan Khash	Sistan Baluchistan	Khash	White	White	Sweet	Local
Jangali Poost Ghermez Roodbar	Gilan	Roodbar	Red	Red	Sweet-sour	Local
Khajei Ghasrodasht Fars	Fars	Shiraz	Pink	White	Sweet	Local
Malas Pishva Varamin	Tehran	Varamin	yellow	White	Sweet	Local
MalasYazdi	Yazd	Yazd	Red	Red	Sweet-sour	Commercial
Makhmal Malas Shahreza	Esfahan	Shahreza	Red	Red	Sweet-sour	Local
Malas No. 1 Saravan	Sistan Baluchistan	Saravan	Yellow	White	Sweet-sour	Local
Poost Nazok Torosh Abarkuh	Yazd	Abarkuh	Red	Red	Sweet-sour	Local
Poost Sefid Dezfoul	Khuzestan	Dezfoul	Yellow-white	White	Sweet-sour	Local
Rabab Poost Ghermez Neyriz	Fars	Neyriz	Red	Red	Sweet-sour	Commercial
Rabab Poost Ghermez Kazeroon	Fars	Kazeroon	Red	Pink	Sweet-sour	Local
Sefid Biardal Borujen	Chahar Mahal-e Bakhtiar	Borujen	Yellwo	Pink	Sour	Local
Shirin Jangal Sisangan	Mazandaran	Sisangan	Red-yellow	Pink	Sweet-sour	Local
Shirin Semnan	Semnan	Semnan	Green-yellow	White	Sweet	Local
Shahsavar Seydan Marvdasht	Fars	Marvdasht	White-yellow	White	Sweet-	Local
Torosh Goli Naz Behshahr	Mazandaran	Behshahr	White-yellow	White	Sour	Local
Torosh Nar Riz Zirab	Fars	Darab	Green-yellow	White	Very sour	Wild

(ANRRC), Yazd Province, Iran. Nineteen pomegranate cultivars were used in the study (Table 1). Two of the cultivars, 'Rabab Poost Ghermez Neyriz' and 'Malas Yazdi', are commercial cultivars widely cultivated in the country. The rest are of local importance in different provinces, except 'Torosh Nar Riz Zirab', a non-commercial (semi-wild type) from the Darab region. The pomegranate cultivars have been planted in a randomized complete block design (RCBD) with three replications per cultivar. Trees were 26-year-old at the time of the experiment and managed following the region's recommended orchard practices.

Measurement of vegetative characteristics

A total of 6 trees per cultivar (3 trees × 3 replicates) was used for vegetative traits measurements. Since the pomegranate trees are trained to the multiple trunk (3-4 trunks) system (the common practice in Iran's commercial orchards), individual trunks diameters were measured at 30 cm above the soil surface and then averaged to get the value for the single trunk diameter. Bark thickness was measured in a small-detached section of bark at 30 cm above the soil surface. Tree height (from the ground level up to the tree peak) and canopy width (in the widest point). Numbers of suckers were simply counted on trees and for shoot angle (°), the insertion of shoots that came directly from the scaffold were measured. In addition, the current year's shoot length (cm) was evaluated after shoot growth stopped (in November) on three scaffolds of trees. Moreover, internode length (cm) was calculated by dividing the current year's shoot length by its corresponded node number.

Measurement of fruit quality attributes

Physical properties. At harvest (which was varied for each cultivar), measurements of fruit physical properties were done on 90 randomly selected fruits per cultivar (10 fruits per tree × 3 trees per replicate × 3 replicate = 90 fruits for each cultivar). Fruits were weighed using a digital balance. Peel and arils were carefully separated manually from the fruit to measure the edible portion. The extracted arils were collected in a tray and mixed thoroughly to assure uni formity.The edible portion of the fruit was determined using the following formula (Ghasemi Soloklui *et al.,* 2019):

Edible portion of fruit (%)=

$$\frac{\text{fresh weight - peel weight - capillary membranes}}{\text{fruit weight}} \times 100$$

Chemicals properties. At harvest, total soluble solids content (TSS) and total acidity (TA) were measured in juice extracted from 90 fruits per cultivar (10 fruits per tree × 3 trees per replicate × 3 replicates = 90 fruits for each cultivar). TSS (in °Brix) was determined using a digital refractometer (model PR-1, Atago, Japan) and TA by titration to pH end-point 8.2 with 0.1 N NaOH and expressed as citric acid equivalent (g CAE100 mL^{-1}) (Horwitz, 1980).

Measurement of stomatal density

In late summer, fully expanded leaves (15 leaves per replication) were collected randomly from the midpoint of the current season's shoots. Stomata numbers were determined using the replica method (Soleimani *et al.*, 2002). The stellate hairs were removed from the lower surface of each leaf using an adhesive tape. A thin film of cellulose acetate was painted directly onto the lower epidermis of the leaf. The cellulose acetate was allowed to dry at room temperature before being peeled from the leaf. Sections were taken from the middle of each leaf. The slides were coded, and a binocular microscope was used for stomatal counts at x 40 magnification. Stomatal density was counted in a field area of one mm^2.

Statistical analysis

Analysis of variances (ANOVA) was performed using SAS version 9.1 (SAS, 2003.). The means were carried out at $P<0.05$ using Duncan's multiple range tests. Correlation between pairs of traits was determined using Pearson's correlation coefficient.

3. Results

Vegetative characteristics

Measured vegetative traits are presented in Table 2 and figure 1. Tree height varied between 1.97 to 4.6 m (Fig. 1). The highest tree height value was observed in 'Khajei Ghasrodasht Fars' (4.6 m), followed by 'Bihaste Ravar' (4.3 m), 'Bihaste Sangan Khash' (4.26 m), and 'Shahsavar Seydan Marvdasht' (4.26 m). 'Malas No. 1 Saravan' showed the smallest tree height (1.97 m) (Fig. 2), some cultivars such as 'Rabab Poost Ghermez Neyriz' and 'Shirin Semnan' categorized as medium height (Fig. 1).

Among the 19 cultivars, the greatest canopy width (4 m) was observed in 'Shahsavar Seydan Marvdasht', while 'Makhmal Malas Shahreza'showed the smallest canopy width (1.50 m) (Fig. 1). 'Khajei

Table 2 - Vegetative tree characteristics of the pomegranate cultivars used in the experiment

Cultivars	Trunk diameter (mm)	Current year shoot length (cm)	Internode length (cm)	Shoot angle (°)	Number of suckers	Bark thickness (mm)
Anar Siah	114 ab	21 bcdefg	3.11 ab	57 defg	18 gh	2.03 b
Bihaste Ravar	98 ab	24.16 b	3.17 ab	60.16 defg	55 bc	2.60 b
Bihaste Sangan Khash	99 ab	17 defg	2.83 abc	53.33 fg	90 cd	2.22 b
Jangali Poost Ghermez Roodbar	78 ab	15.66 fg	2.57 abc	63.33 defg	191 bcd	2.83 b
Khajei Ghasrodasht Fars	121.33 a	22 bcde	3.39 a	65 def	8.33 h	2.56 b
Malas Pishva Varamin	97.67 ab	16.33 efg	3.17 ab	65 def	161 abc	1.82 b
Malas Yazdi	74.67 ab	23 bcd	2.14 c	46.66 g	70 efg	2.46 b
Makhmal Malas Shahreza	77 ab	26.50 b	2.05 c	86.66 ab	180 abc	1.77 b
Malas No. 1 Saravan	61.0 b	17.6 cdefg	1.96 c	60 defg	22 gh	1.70 b
Poost Nazok Torosh Abarkuh	93.83 ab	41.50 a	3.39 a	63.33 defg	70 efg	3.24 a
Poost Sefid Dezfoul	89 ab	15 g	2.39 bc	73.33 abcde	93 def	1.64 g
Rabab Poost Ghermez Neyriz	110 ab	21 bcdefg	2.52 abc	56.66 efg	143 bcd	2.23 bcdefg
Rabab Poost Ghermez Kazeroon	107.17 ab	16.33 efg	2.33 bc	83.33 abc	1 h	1.62 g
Sefid Biardal Borujen	111.33 ab	23.50 bc	2.61 abc	86.66 ab	215 a	1.55 g
Shirin Jangal Sisangan	73.76 ab	17.6 cdefg	2.01 c	68.33 cdef	89 def	2.10 cdefg
Shirin Semnan	99 ab	16.83 defg	2.80 abc	75 abcd	126 def	2.92 ab
Shahsavar Seydan Marvdasht	114 ab	21.3 bcdef	3.17 ab	70 bcdef	35 fgh	2.64 abcd
Torosh Goli Naz Behshahr	67.75 ab	17.6 cdefg	2.33 bc	90 a	35 fgh	2.40 bcdef
Torosh Nar Riz Zirab	62.17 b	21.3 bcdef	2.52 abc	66.66 cdef	69 efg	2.42 bcdef

Similar letters in each column indicate non-significant differences among cultivars at P≤0.05.

Ghasrodasht Fars' had the greatest single trunk diameter (121.33 mm), whereas 'Malas No.1 Saravan', had the lowest single trunk diameter (61 mm) (Table 2).

The longest current year's shoot length (41.50 cm) was recorded in 'Poost Nazok Torosh Abarkuh', while the shortest (15 cm) obtained in 'Poost Sefid Dezfoul' (Table 2). Internode length varied from 1.96 cm ('Malas No. 1 Saravan') to 3.39 cm ('Khajei Ghasrodasht Fars') (Table 2). The correlation between internode length and tree height was also statistically significant (r=0.55; p=0.0001) (Fig. 3). Shoot angle was within the range of 46.6 to 90 (Table 2). Significant differences were observed in the number of suckers among the pomegranate cultivars. The highest number of suckers per tree was recorded in 'Sefid Biardal Borujen' (215), while the lowest number was counted in 'Rabab Poost Ghermez Kazeroon'. In addition, the highest (3.24 mm) and lowest (1.55 mm) bark thickness were recorded in 'Poost Nazok Torosh Abarkuh' and 'Sefid Biardal Borujen', respectively (Table 2).

Considering all vegetative characteristics, in particular, tree height, trunk diameter and internode length; cultivars could be classified into four groups,

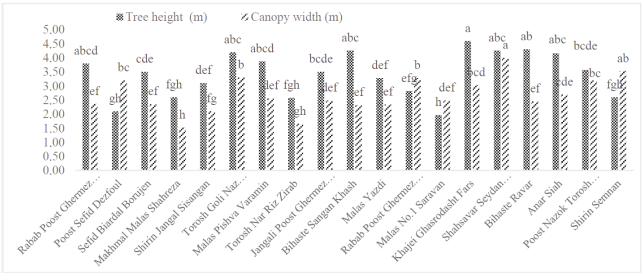

Fig. 1 - Tree height and canopy width in 19 Iranian pomegranate cultivars. Similar letters indicate non-significant differences among cultivars (P≤0.05).

Fig. 2 - Comparison of tree height between a vigorous pomegranate cultivar (A) and 'Malas No. 1 Saravan' (B), the most dwarf cultivar in the pomegranate genotypes studied.

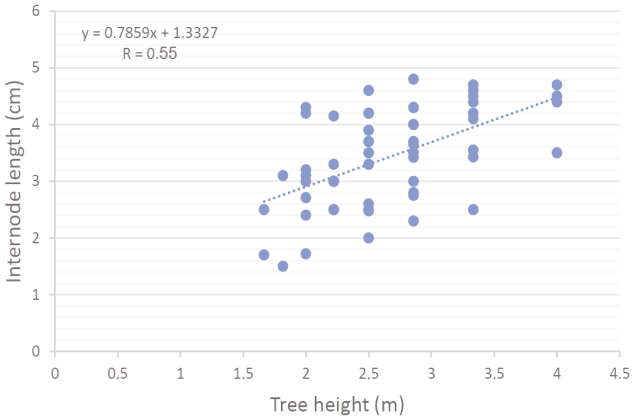

Fig. 3 - Pearson's correlation coefficients between internode length and tree height in 19 Iranian pomegranate cultivars.

1) vigorous cultivars: 'Khajei Ghasrodasht Fars', 'Shahsavar Seydan Marvdasht', 'Bihaste Ravar', 'Bihaste Sangan Khash', 'Torosh Goli Naz Behshahr' and 'Anar Siah', 2) Semi-vigorous cultivars: 'Malas Pishva Varamin', 'Rabab Poost Ghermez Kazeroon', 'Poost Nazok Torosh Abarkuh', 'Jangali Poost Ghermez Roodbar', 'Sefid Biardal Borujen', 'Malas Yazdi' and 'Shirin Jangal Sisangan', 3) Semi-dwarf cultivars: 'Rabab Poost Ghermez Neyriz', 'Shirin Semnan', 'Makhmal Malas Shahreza' and 'Poost Sefid Dezfoul', and 4) Dwarf cultivars: 'Torosh Nar Riz Zirab' and 'Malas No. 1 Saravan'.

Stomata density

As shown in figure 4, a large variation in stomatal density (from 46.91 to 108.91 stomata per mm^2) was observed among studied cultivars. 'Shirin Semnan', showed the highest stomatal density, while 'Shahsavar Seydan Marvdasht' had the lowest stomatal density. Results of Pearson correlation analysis provide significant negative correlations between stomatal density and tree height (r = -0.44; *P* =0.0005) (Fig. 5).

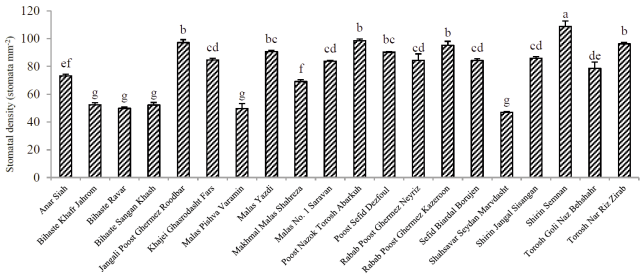

Fig. 4 - Frequency distribution of stomatal density in 19 Iranian pomegranate cultivars. Bars indicate SE (n=45).

Fruit quality traits

The highest fruit weight was perceived in 'Shahsavar Seydan Marvdasht' (378.17 g), followed by 'Malas Yazdi' (230.83 g) and 'Jangali Poost Ghermez Roodbar' (214.67 g), while the smallest fruit (62.17 g) was observed in 'Torosh Nar Riz Zirab' (a semi-wild cultivar) (Table 3). The percentage of the edible portion of the fruit ranged from 49.80 (in 'Torosh Nar Riz Zirab') to 71.98% (in 'Biardal Borujen') (Table 3). The highest (18.7°Brix) and lowest (13.0°Brix) TSS was measured in 'Sefid Biardal Borujen' and 'Shahsavar Seydan Marvdasht', respectively (Table 3). Fruit juice pH varied from 3.13 to 4.43 among the studied pomegranate cultivars, with the minimum and maximum pH measured respectively in 'Torosh Nar RizZirab', and 'Anar Siah'. Moreover, the highest and lowest TA were observed in 'Torosh Nar Riz Zirab' (8.47 g CAE 100 mL^{-1}) and 'Bihaste Sangan Khash' (0.50 g CAE 100 mL^{-1}), respectively.

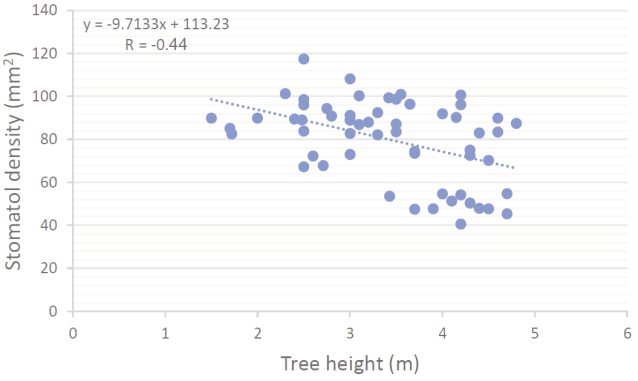

Fig. 5 - Pearson's correlation coefficients between stomatal density and tree height in 19 Iranian pomegranate cultivars.

4. Discussion and Conclusions

The results of this research present a wide range of vigor and dwarfing potential in Iranian pomegranates. In high-density orchards, controlling tree vigor and canopy size is important for enhancing the orchard efficiency and productivity (Umar and Sharma, 2008). Vegetative growth can be defined by several parameters such as; total shoot length, internode length, number of terminals and lateral shoot, and trunk cross-sectional area (Weibel *et al.*, 2003). In classifying trees according to size, dwarf trees are approximately 2.5 m or less in height when mature (Castle, 1992). Considering the above men-

Table 3 - Fruit characteristics of the pomegranate cultivars used in the experiment

Cultivars	Fruit weight (g)	Edible portion (%)	TSS (°Brix)	pH	TA (%)
Anar Siah	124.0 ef	53.31 cd	13.8 hi	4.43 a	0.70 hi
Bihaste Ravar	140.32 e	54.30 cd	14.0 fghi	4.06 cd	0.55 i
Bihaste Sangan Khash	156.0 de	60.39 abcd	13.83 ghi	4.38 ab	0.50 i
Jangali Poost Ghermez Roodbar	214.67 b	61.88 abcd	18.33 ab	3.49 hij	2.17 efg
Khajei Ghasrodasht Fars	207.50 bc	59.53 abcd	14.66 efghi	3.65 gh	2.51 def
Malas Pishva Varamin	131.0 ef	67.81 ab	15.5 defgh	3.89 def	1.41 ghi
MalasYazdi	230.83 b	49.80 d	16.0 cdef	3.70 fgh	1.85 fg
Makhmal Malas Shahreza	133.50 ef	60.32 ab	17.66 abc	4.2 bc	0.6 i
Malas No. 1 Saravan	125.83 ef	61.52 abcd	15.33 defgh	3.34 ijk	3.30 cd
Poost Nazok Torosh Abarkuh	129.50 ef	62.66 abcd	16.0 cdef	3.49 hij	2.8 de
Poost Sefid Dezfoul	88.0 fg	57.94 bcd	16.66 bcde	4.16 bc	0.88 hi
Rabab Poost Ghermez Neyriz	190.0 bcd	56.61 bcd	14.83 efghi	3.53 hi	2.57 def
Rabab Poost Ghermez Kazeroon	161.67 cde	54.16 cd	15.8 cdefg	3.84 efg	1.55 k
Sefid Biardal Borujen	159.33 de	71.98 a	18.66 a	3.28 jk	3.97 c
Shirin Jangal Sisangan	192.67 bcd	55.10 bcd	15.5 defgh	4.04 cde	1.35 ghi
Shirin Semnan	158.0 de	62.01 abcd	14.0 fghi	4.16 bc	0.70 hi
Shahsavar Seydan Marvdasht	378.17 a	56.52 bcd	13 i	4.16 sm	0.61 i
Torosh Goli Naz Behshahr	126.83 ef	64.67 abc	17.16 abcd	3.14 k	5.47 b
Torosh Nar Riz Zirab	62.17 g	60.77 abcd	16.66 bcde	3.13 k	8.47 a

Similar letters in each column indicate non-significant differences among cultivars at P≤0.05.

tioned vegetative attributes and keeping in mind the Castle (1992) scale, 'Malas No. 1 Saravan' and 'Torosh Nar Riz Zirab' were the most dwarf size, about half that of vigorous cultivars. Thus, this cultivar has potential to be used directly as dwarfing pomegranate rootstocks, although a more detailed study on propagation, graft compatibility, and tolerance to biotic and abiotic stress will shed more light on the suitability of these cultivars as rootstock. As a dwarfing source, these cultivars could be utilized as parents in breeding programs to develop superior dwarf scion and rootstock cultivars.

This study demonstrated that internode length was associated with tree size. In general, some cultivars such as 'Makhmal Malas Shahreza', 'Poost Sefid Dezfoul' and 'Malas No. 1 Saravan' have the smallest tree sizes and shorter internodes lengths than other cultivars. The average internode length depended on the number of nodes per extension unit (Costes and Garcia-Villanueva, 2007). Dwarf trees usually produce very short internodes length, resulting in branches more compact than vigorous trees (Ingels *et al.*, 2002). Obtained results for internode length are in agreement with those of Murase *et al.* (1990), on peach trees grafted on dwarfing rootstocks that had shorter internodes than trees grafted on vigorous rootstock. The results of the current study also showed a wide variation in sucker production among cultivars. In this regard, cultivars such as 'Rabab Poost Ghermez Kazeroon' and 'Khajei Ghasrodasht Fars' with the lowest number of suckers have the advantage of easy management and also may be suitable for preferred single trunk training system in modern fruit orchards.

Stomata are directly responsible for the trade-off between water loss and carbon acquisition (Raven, 2002). Stomatal density as a quantitative attribute is genetically determined (Gailing *et al.*, 2008). Some plant species have been reported as possessing generally high heritability (i.e., less dependence on environmental conditions) in their stomatal traits (Sharma and Dunn, 1969; Orlovic *et al.*, 1998). Drogoudi *et al.* (2012) reported stomatal density among four pomegranate cultivars ranging from 68 to 149.9 stomata mm^{-2}. Also, Meena *et al.* (2011) reported astomatal density of 130.67 stomata mm^{-2} for pomegranate. These results are following the findings of the current study, and minor differences in the results could be due to cultivar or climate differences. Interestingly, some of the vigorous cultivars such as 'Bihaste Ravar', 'Shahsavar Seydan Marvdasht' and 'Bihaste Sangan Khash' had low

stomatal density (between 46 to 52 stomata mm^{-2}), whereas dwarf and semi-dwarf cultivars, including 'Shirin Semnan', 'Torosh Nar Riz Zirab' and 'Poost Sefid Dezfoul' possess very high stomatal density (108, 96 and 90 stomata mm^{-2}, respectively). These results are in line with the findings of Barrientos-Pérez and Sanchez-Colín (1982), who reported that stomatal density could be a good method to classify the growth habit in avocado trees (Barrientos-Pérez and Sánchez-Colín, 1982).

Thus, the data on fruit attributes would provide useful information for selecting the best dwarf cultivars to be used directly as scion cultivars on their root or as parent materials in scion cultivars breeding programs. The evaluation of pomegranate fruit quality (physical and chemical) in the local material has previously been carried out in Iran (Akbarpour *et al.*, 2009), Turkey (Özkan, 2001), Italy (Barone *et al.*, 2001), and Greece (Drogoudi *et al.*, 2005). Tehranifar *et al.* (2010) described important fruit traits of 20 pomegranate cultivars from different regions in Iran. They found that the fruit weight, peel percentage, aril percentage, and juice percentage were within the range of 196.89-315.28 g, 32.28-59.82%, 37.59-65% and 26.95-46.55%, respectively, which are in line with the results of the current study. Moreover, Yıldız *et al.* (2003) reported that promising pomegranate genotypes, selected from Hizan (Bitlis) in Turkey, had 192.3-388.3 g fruit weight, 28-55% juice percentage, 0.33-4.03% juice acidity and 10.0-17.0% juice soluble solids content. On the other hand, Mars and Marrakchi (1999) defined fruit characteristics of 30 pomegranate genotypes from Tunisia. They reported fruit weights ranging from 196.1 to 673.6 g, pH from 2.9 to 4.6, soluble solid contents from 13.3 to 16.9°Brix, and acidity from 0.2 to 3.1 g CAE 100 mL^{-1}. Consequently, the pomegranate studied herein had many similarities to those described in other studies concerning fruit traits such as fruit weight, soluble solids content, pH and acidity. Minor differences in these traits across the studies could arise from different plant materials and varied climatic conditions. In this study edible portion was between 49.80 to 71.98%; whereas, Al-Maiman and Ahmad (2002) reported an edible portion of about 55-60% of the total fruit weight. This study showed that most cultivars except 'Torosh Nar Riz Zirab' and 'Poost Sefid Dezfoul' have big and medium sized fruits.

In general, considering vegetative characteristics and fruit quality attributes, 'Rabab Poost Ghermez Neyriz' a commercial cultivar with semi-dwarfing growth habit and good fruit quality (have big fruits, with high TSS contents and low acidity) is a promising candidate for establishing high-density orchards on its roots. Moreover, some cultivars such as 'Shirin Semnan', 'Makhmal Malas Shahreza' and 'Malas No. 1 Saravan', which categorized as dwarfing or semi-dwarfing cultivars and possessed quite good fruit quality, have the potential to be used as a parent in breeding programs to develop dwarf pomegranate cultivars or dwarfing rootstocks. 'Torosh Nar Riz Zirab' is a semi-wild cultivar with small tree size (dwarf cultivar) but represents poor fruit quality attributes. Thus, this cultivar can be considered as a dwarfing rootstock in pomegranate production. However, a more detailed study on propagation, graft compatibility, and tolerance to biotic and abiotic stress will shed more light on these cultivars' potentials as rootstock.

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Short note

Stable growth inhibition of potted fig (*Ficus carica* L.) trees by soil sickness

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Key words: planting timing, root enclosing, rooted cutting size, shoot growth.

Abstract: The study was conducted to know a damage progress of soil sickness of fig trees and effect of initial planting conditions on it. Shoot growth of 10-liter potted 'Masui Dauphine' figs was inhibited with sick soil from the 1st year of planting, and a stable dwarfish growth was maintained form the 2nd to 9th years, with only a few trees dying. The sick soil affected trees planted in 25-liter pots in June worse than those planted in February, and trees with roots enclosed by non-woven fabric worse than without it. However, these differences had faded by the 3rd year. The sick soil affected trees in 60-liter pots in the 1st year of planting worse in smaller rooted cuttings than in larger ones. However, in subsequent years, growth inhibition was not affected by the rooted cutting size. These results suggest that the initial conditions, such as planting timing, physical barriers to rooting, and rooted cutting size, all affect potted fig tree growth in the early growing period, and influence the observed damage caused by sick soil. However in subsequent years, dwarfish growth in sick soil may attain a stable level, which is maintained for many years with very low mortality.

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The authors declare no competing interests.

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1. Introduction

In the Japanese fig industry, the common-type fig 'Masui Dauphine' ('San Piero' sensu Condit, 1955) is a major cultivar and its second crops is sold along with fresh fruits. The fig is regarded as an easily cultivable fruit tree; however, some obstacles exist. An extreme decline in tree vigour, so called "soil sickness", has long been a serious obstacle for fig culture in Japan (Hirai and Nishitani, 1953; Hosomi, 2011). Although the exact cause of soil sickness is still unclear, there exist several causal hypotheses, e.g., toxic chemicals (Hatsuda *et al*., 1960), nematode pests (Condit, 1947; Sato and Shichijo, 1953), and some soil-borne diseases (Hosomi and Uchiyama, 1998). It is a common characteristic that some trees in a field will begin to weaken within a few years after planting and that normal growth cannot be expected merely by replanting (Hirai and Nishitani, 1953). However, it is unknown whether soil sickness damage increases year by year and finally kills the trees, or if the soil does not degenerate further and sometimes recovers. In other fruit, it is known that rooted

cutting size influences growth inhibition by sick soil (Iwasaki, 1962; Hirano, 1968), but it is also unknown for fig trees whether such initial planting condition influences subsequent inhibition by sick soil for long periods.

In the present study, yearly changes in shoot growth were investigated using potted 'Masui Dauphine' fig trees with sick soil. Whether the timing of planting, physical disturbing of rooting or the size of rooted cuttings influence growth inhibition by sick soil, have also been investigated. The results will aid in recognition of the infestation process of soil sickness, and give information for a replanting plan of cultivated fig trees.

2. Materials and Methods

Yearly shoot growth of potted fig trees with sick soil (Exp. 1)

Every April from 1990 to 1994, the rooted cuttings of 'Masui Dauphine' figs were planted in 10-liter unglazed pots filled with a mixture of sandy soil, Kanuma soil and vermiculite (1:1:1, v/v/v). These were grown for 9 years in the open. In each June of the 1st planting year, 500 milliliter (ml) of the stored sick soil, which had been collected from fig orchards exhibiting the symptoms of soil sickness (Hosomi and Uchiyama, 1998), were added to the bed surface of 4 to 8 trees (28 trees in total). Four to 5 trees in each planting year (23 trees in total) were controls. Each surface of the bed (including control) was mulched with 500 ml of peat moss. Fifty ml of slow-release fertilizer (100 days type N: P_2O_5: K_2O=16: 5:10 plus micronutrients) was applied each June. Irrigation was applied automatically to prevent soil drying. One shoot per tree was elongated and the other shoots were disbudded. Each March, the shoots were pruned above their 2nd or 3rd node from the base and the dry weight was measured.

Growth inhibition by soil sickness under conditions of planting timing with/without physical barriers to rooting (Exp. 2)

In December 1999, 18 rooted cuttings of 'Masui Dauphine' figs was raised in individual bag (22×5 cm) with vermiculite under the greenhouse heated at 15-25°C. The bags were made of non-woven fabric, which had been treated by cupric hydroxide (Spin Out; Griffin Co.) to prevent root spiraling. Two shoots per rooted cutting were elongated. In February 2000 the rooted cuttings were removed from the bag and planted in 25-liter plastic pots filled with a mixture of Kanuma soil and vermiculite (1:1, v/v). Each 2.3-liter root area in 6 rooted cuttings was enclosed with non-woven fabric (polylactic acid span-bond 100 g/m^2) at planting. Two-liter of the stored sick soil in experiment 1, was added to each bed surface at 25 days after planting. The sick soil was also added to each bed of other 6 rooted cuttings without enclosing the roots. The remaining 6 rooted cuttings were controls. Soon after the sick soil addition, each surface of the beds (including controls) was mulched with 1.5- to 2-liter of peat moss. The other 36 rooted cuttings were likewise raised by cuttings from April 2000, and were planted in 25-liter plastic pots in June 2000. At that time 12 rooted cuttings had the root areas enclosed and were added with sick soil, a further 12 rooted cuttings were added with sick soil alone, and the other 12 were controls.

The trees were grown for 3 years in unheated greenhouse ventilated by roof and side vents. One hundred ml of slow-release fertilizer in experiment 1 was applied to each tree in March (February planting) or July (June planting) in the 1st year, and in June in the 2nd and 3rd year. Irrigation was applied automatically to prevent soil drying. Only 2 shoots per tree were elongated, and all other shoots were disbudded each year. Each March from 2001, the shoots were pruned above their 2nd or 3rd node from the base and the dry weight was measured.

Growth inhibition by soil sickness under conditions of different rooted cutting size (Exp. 3)

From April 1998, the rooted cuttings of 'Masui Dauphine' figs were raised by cuttings in 1.3-liter, 10-liter and 18-liter plastic pots filled with vermiculite with 6.5 ml, 50 ml and 90 ml respectively of slow-release fertilizer in experiment 1. The inner walls of these pots had previously been painted with cupric hydroxide (Spin Out; Griffin Co.) to prevent root spiraling. One shoot per tree was elongated and all other shoots were disbudded. In June 1999, the shoots were pruned and their length, basal diameter and dry weight was measured. I also took the rooted cuttings from the pots, washed off the soil, and calculated the root volume from the loss in weight when the roots were dipped in water. Thus, each of 10 rooted cuttings in 3 size categories (S, M and L) were prepared as in Table 1 and were planted to 60-liter plastic pots filled with a mixture of Kanuma soil and vermiculite (1:1, v/v). Two weeks after planting, 5-liter of the stored sick soil in experiment 1 was added to the bed surface of 6 trees in each category. Four

Table 1 - Rooted cuttings used in the test examining the effect of its size on sick soil damage

Size categories	Silck soil inoculation [z]	Shoot [y]			Root volume [x]	
		Length (cm)	Basal diameter (mm)	Dry weight (g)	In each group (cm^3)	In each size (cm^3)
Large (L)	+	116.5±5.5 [w]	15.8 ± 0.4	43.5±2.4	369.7±63.4	357.6
	-	126.5±6.9	15.3 ±0.7	40.7±5.8	339.5±65.9	
Medium (M)	+	107.8±5.0	14.4±0.5	33.4±3.2	296.8±32.9	280.2
	-	116.8±4.8	14.0±1.0	33.8±4.4	255.3±53.2	
Small (S)	+	65.5±2.1	11.4±0.2	11.1±0.4	148.5±26.1	168.2
	-	66.5±4.3	12.8±0.3	13.0±1.3	197.8±20.9	

[z] Yes (+) or No (-) of scheduled sick soil inoculation.
[y] Only one shoot per rooted cutting was elongated, pruned before planting and its size was measured.
[x] Estimated from the loss in rooted cutting weights when dipping roots in water.
[w] Mean±SE.

trees in each category were controls. Each surface of the beds (including controls) was mulched with 1.5-liter of peat moss, and all trees were grown for 4 years in the open. Three hundred ml of slow-release fertilizer in experiment 1 was applied each June. Irrigation was applied automatically to prevent soil drying. Three shoots per tree in the 1st year, and 6 shoots in subsequent years, were elongated and all other shoots were disbudded. Each March the shoots were pruned above their 2nd or 3rd node from the base and dry weight was measured.

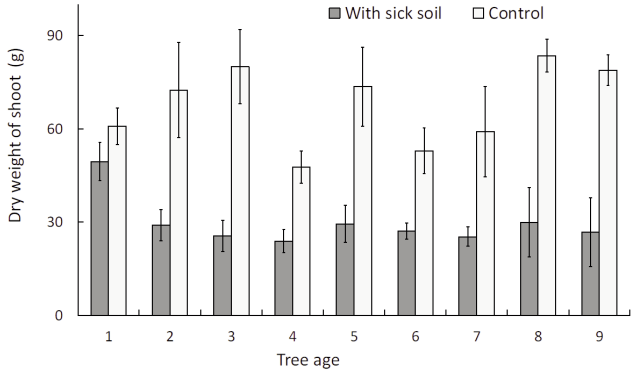

Fig. 1 - Yearly change in dry weight of shoot per 'Masui Dauphine' fig trees grown in 10-liter potted with/without sick soil. Vertical bars indicate SE.

3. Results and Discussion

Yearly shoot growth of potted fig trees with sick soil

The results are shown in figure 1. For the control trees, the average dry weight of shoot (shoot weight) varied in the range of 48 to 80 g. For trees with sick soil, the shoot weight was 49 g (81% of control) in the 1st year, decrease in the 2nd year and was maintained the range of 24 to 29 g (32 to 50% of control) in subsequent 8 years. Only 2 trees died in the sick soil during the testing period. In other word, the sick soil, added to bed soil, inhibit growth of 10-liter potted fig trees from the 1st planting year. It takes 1 year to confirm the growth inhibition, after which sick soil cause stable dwarf growth for many years without trees dying.

Growth inhibition by soil sickness under conditions of planting timing with/without physical barriers to rooting

The results are shown in figure 2. For control trees, the average dry weight of total shoot per tree

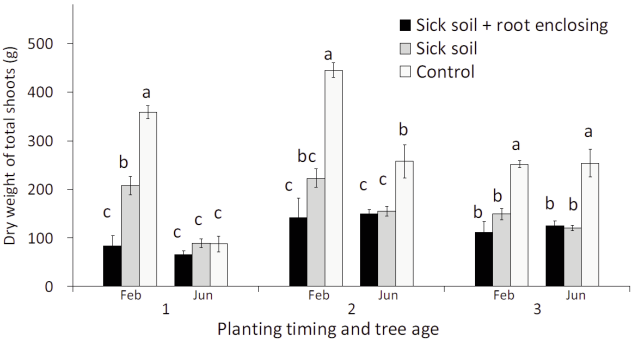

Fig. 2 - Dry weight of total shoot per 'Masui Dauphine' fig trees grown in 25-liter pots with/without sick soil under conditions of planting timing (February and June) with/without root-enclosing by non-woven fabric. Vertical bars indicate SE. Different letters indicate significance among conditions in each age at 5% level by Tukey-HSD test using R project 3.6.1.

(total shoot weight) varied in the range of 88 to 445 g, and the values of the trees planted in June was apparently less than those planted in February in the 1st and 2nd years, and equalized with them in the 3rd year.

For trees with sick soil, the total shoot weight varied in the range of 89 to 223 g, less than in controls. These values for June planted trees were less than for February planted ones in the 1st year, but were equalized form the 2nd to the 3rd year. For trees with sick soil plus root-enclosing by non-woven fabric, the total shoot weight varied in the range of 66 to 149 g. In early years for February planting trees, inhibited growth by sick soil plus root-enclosing tended to be less than those with sick soil alone. Uchida *et al.* (1998) reported that root wrapping materials such as woven flax disturbed the initial root growth of deciduous trees (*Magnolia kobus* and *Quercus acutissima*) for up to 10 months after planting. In this experiment the fabric may also disturb root elongation before rooting out, and may act as a growth inhibitor in the early growing period.

For June planting trees, however, no effect to the corresponding control was detected in sick soil application in the 1st year, and in root-enclosing in all tested years. Hirano (1968) reported that, in peach *Prunus persica* Batsch trees, growth inhibition by sick soil infestation was greater when vigorous growth was expected. It seems that the damage of sick soil and root-enclosing of this study were masked under delayed growth conditions due to the late planting.

Growth inhibition by soil sickness under conditions of different rooted cutting size

The results are shown in figure 3. For control trees, the dry weight of total shoot per tree (total shoot weight) was about 125 g in the 1st year and did not differ between the rooted cutting size (L, M and S). After the 2nd year the values increased, and varied in the rage of 230 to 336 g with a tendency to be greater in size-S. Ishimaru *et al*. (2003) reported that a smaller size of the rooted cuttings cause high relative initial growth rate in 3 species of broad-leaved tree (*Quercus serrata*, *Q. glauca* and *Myrica rubra*). The smaller rooted cuttings in this experiment also increase shoot vigour in the early period, and overcome the initial handicap of the tree biomass.

For trees with sick soil, the total shoot weight was about 107 g (86% of control) in rooted cutting size-L and M, and 70 g (56% of control) in size-S in the 1st year. Values were about 73 g in the 2nd year, 169 g in the 3rd year and 196 g in the 4th year, and apparently less than in controls, but did not differ between the size categories. In other word, the smallness of the rooted cutting intensified the sick soil damage in the planting year. However after the 2nd year from planting, that damage intensification and the vigour of small rooted cuttings compensate for each other and the shoot growth of every group with sick soil equalized. It had been reported that growth inhibition by sick soil is severe in smaller rooted cuttings in satsuma mandarin *Citrus unshiu* (Iwasaki, 1962) and peach (Hirano, 1968). My results are not inconsistent with these reports, because the phenomena they report were observed only in the 1st planting year.

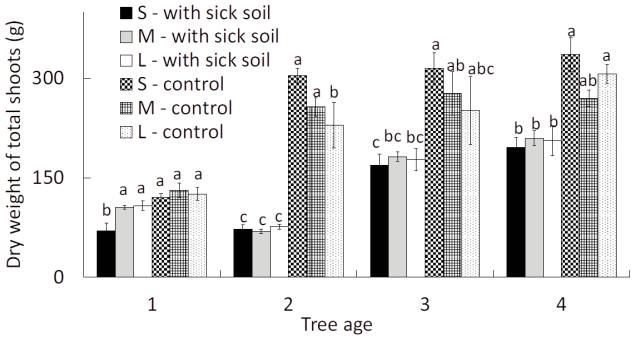

Fig. 3 - Dry weight of total shoot per 'Masui Dauphine' fig trees grown in 60-liter pots with/without sick soil under conditions of different rooted cutting size. The rooted cuttings in 3 size categories (S, M and L) were prepared as in Table 1. Vertical bars indicate SE. Different letters indicate significance among conditions in each age at 5% level by Tukey-HSD test using R project 3.6.1.

The results of my 3 experiments suggest that tree growth with sick soil may stabilize to a particular level determined by the pot size. Longer times may be required for stabilization in larger pots with sick soil: One year in experiment 1 (10-liter pots), over 2 years in experiment 2 (25-liter pot) and over 3 years in experiment 3 (60-liter pot). Probably the longer terms for stabilization are because larger pots take longer to full with roots. The initial conditions, such as the timing of planting, physical barriers to rooting, and rooted cutting size, affect tree growth and observed damage due to soil sickness for the early planting period. However, the initial delays in growth made up, and did not determine future growth inhibition by sick soil. Hosomi and Uchiyama (1998) reported that parasitization by some microorganisms are most important factors in sick soil in fig orchards. Stable inhibition of soil sickness may be because root regeneration activity in certain restricted root zone balances the damage caused by these parasites. In

peach orchards, Yamada and Ono (1970) observed that the trees grew for 7 years with a constant difference between with and without sick soil. In fig orchards, it is also estimated that a stable dwarfish growth is maintained for many years in sick soil. I can expect an effect to overcome the problem in a methods to accelerate tree growth permanently, for example a vigour root stock (Hosomi *et al*., 2002), and are unable to expected it in devising the methods of planting. All results in present study use potted trees in which rooting is restricted physically by the pot wall. Field tests with free rooting and sick soil are needed to learn more about these competing effects.

4. Conclusions

Dwarfish growth by sick soil inoculation in potted 'Masui Dauphine' figs was maintained form the 2nd to 9th years after inoculation, resulting in few deaths. The initial planting conditions, such as planting timing (February and June), non-woven fabric as physical barriers to rooting, and rooted cutting size, all influence the observed damage caused by sick soil in the initial growth of those potted trees. However, in subsequent years, their growth converged to similar levels of weakness. A certain and stable growth inhibition on figs over many years, irrespective of planting condition, seems to be a basic characteristic of soil sickness in fig culture.

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