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Influence of ground cover and tunnels on production of Red Russian kale in urban gardens

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Abstract: Kale, *Brassica oleracea* L. var. *acephala*, is an important urban crop for human health and may potentially be grown year-round even in a temperate climate. We investigated black plastic and straw mulch compared to bare soil cover in low tunnels at 10 urban garden sites and in low tunnels within a high tunnel in the USA to ascertain the influence on yield and nutrients of Red Russian kale, soil temperature, air temperature, weed pressure, and aphid abundance. Kale had low yield in garden sites, likely because the outside environment was too cold for low tunnels to gain and retain heat. Cultivating kale in a high tunnel resulted in good yields, especially when paired with a low tunnel and plastic or straw mulch, which resulted in the highest air and soil temperatures. The amount of minerals in plants within the high tunnel largely did not vary across combinations of low tunnels and ground covers, except for copper and sulfur, which were lowest in plots with no low tunnel or ground cover. Also, dietary fiber was higher when no low tunnel or ground cover was used compared to plots with a low tunnel and no ground cover. Weeds were suppressed by straw and black plastic mulch, but none of the ground covers influenced aphid abundance. Overall, our work demonstrates that Red Russian kale can be grown in a temperate climate during winter with some combinations of tunnels and ground covers.

1. Introduction

As urban agriculture becomes more common worldwide, analyses of its benefits, limitations, and role within sustainable cities and the food supply chain also become more common (Orsini *et al.*, 2013; Goldstein *et al.*, 2016; Azunre, 2019; Taylor *et al.*, 2021). Urban agriculture is not a replacement for large-scale rural agriculture partly because it cannot meet caloric needs of the growing and urbanizing human population. However, urban agriculture can address nutritional deficiencies in humans by providing a local source of crops that provide important micronutrients (Weidner *et al.*, 2019). Horticultural crops, such as pulses

(Cerozi *et al.*, 2022), tubers (Richardson and Arlotta, 2023), root crops (Cerozi *et al.*, 2022), and vegetables (Song *et al.*, 2020) that are nutrient-dense, high-yielding, and easy to cultivate, are among the most suitable crops for urban production (Clinton *et al.*, 2018). Production of horticultural crops in urban areas also shifts dietary intake toward consumption of locally grown fresh foods (McCormack *et al.*, 2010), which has health benefits, such as preventing chronic disease (Boeing *et al.*, 2012). Production of horticultural crops in urban areas can also potentially have numerous social and environmental benefits, such as providing educational spaces, connecting people with the food supply chain, reducing food miles, and efficiently using natural resources (Specht *et al.*, 2014).

Kale, *Brassica oleracea* L. var. *acephala*, is one such crop well-suited to urban areas. It is grown for its high yield of edible leaves that are consumed raw or cooked and is an important staple crop for diversified market farms, community supported agriculture, and school, community, and home gardens in urban areas. Brassicaceous crops, such as kale, are a good source of mineral nutrients, antioxidant phytochemicals, secondary metabolites, and prebiotic carbohydrates (Manchali *et al.*, 2012; Chang *et al.*, 2019). Kale is higher in calcium, folate, vitamin K, riboflavin, and vitamin C than other brassicaceous crops. Furthermore, kale is a good source of vitamins (A, B1, B2, B6, C and E), folic acid and niacin, fatty acids, and essential minerals (especially K, Ca, Mg, Fe and Cu) (Jahangir *et al.*, 2009; Acikgoz, 2011; Thavarajah *et al.*, 2016). Young kale sprouts especially contain a high amount of protein and dietary fibers (Vale *et al.*, 2015).

Consumption of kale is linked to several health benefits and it has traditionally been used to treat various diseases, such as gastritis and gastric ulcer (Leonti and Casu, 2013), diabetes mellitus, rheumatism, bone weakness, ophthalmologic problems, hepatic diseases, anemia, and obesity (Lemos *et al.*, 2011; Gonçalves *et al.*, 2012; Kuerban *et al.*, 2017). Kale consumption has shown promising results in prevention of cancers of the reproductive system (Han *et al.*, 2014), gastrointestinal system, and lungs (Wu *et al.*, 2013). The strong inhibitory activities of kale extracts against glioblastoma cells and liver cancer cells, but negligible effects on the metabolites and bioactivity of normal cells, demonstrate its antiproliferative properties against tumorous cells (Gonçalves *et al.*, 2012). Furthermore, kale extracts demonstrate antigenotoxicity: properties that maintain the genetic information within a cell by preventing damage and mutations (Gonçalves *et al.*, 2012). Also, people with hypercholesterolemia who consumed 150 mL of kale juice daily for 12-weeks significantly increased their HDL-cholesterol and HDL- to LDL-cholesterol ratio and significantly reduced their LDL-cholesterol (Kim *et al.*, 2008). Kale can be considered a functional food since it may be useful for preventing different chronic degenerative diseases.

Kale is relatively easy to grow, cold hardy, and can provide a steady supply of fresh greens for most of the year if practices for extended season and winter production in temperate climates are identified. Extended season and winter production rely, in part, on protective structures (e.g., low tunnels, high tunnels) that trap solar radiation as heat in air, soil, and water. These structures are common in urban temperate climates and may provide a suitable environment for crop growth when temperatures are otherwise too low (Lamont, 2009). Protective structures act to reduce the frequency and duration of freezing temperatures that would otherwise kill crops. Exposure to freezing temperatures can cause marginal necrosis on kale leaves and temperatures between -14° to -30°C can kill the plant. Protective structures not only serve to reduce the frequency and duration of freezing temperatures but should also optimize the daily duration of temperatures above the threshold for growth. The optimal temperature range for kale growth is 16°-21°C, but growth is possible above 4.4°C (Andrews and Coop, 2011).

Mulches can also be a possibility for aiding in season extension or winter production. Organic mulch, like straw or woodchips, may have insulating properties (Richardson *et al.*, 2023) that slow the rate of heat loss in protective structures each night and slow the warming each day. Therefore, organic mulches may reduce both the frequency and duration of freezing events as well as the duration of optimal growing conditions. Black plastic mulch is known to warm soil, but it is unclear whether this material will act to buffer temperatures to the same extent as organic mulches. In northern climates where freezing temperatures are the norm, it is recommended that crops be grown on bare ground to allow a rapid buildup of heat within structures during the day (Coleman, 2009), but the influence of mulches on kale in low and high tunnels is not completely known.

Providing plants with optimal environmental conditions improves their rate of growth and yield but

may also support the growth and development of weeds and insect pests that negatively affect yield. Lepidopteran eggs laid in the summer or fall will yield caterpillars feeding in an extended season crop. Aphids will continue to thrive in winter production because the same conditions that are optimal for kale growth are sufficient for aphid survival and reproduction. Aphid pest species, such as green peach aphid (*Myzus persicae* Sulzer), potato aphid (*Macrosiphum euphorbiae* Thomas), and cabbage aphid (*Brevicoryne brassicae* L.), have been reported to continue asexual reproduction at temperatures as low as 5-10°C (Barlow, 1962; Soh *et al.*, 2018).

We researched combinations of protective structures and ground covers for production of Red Russian kale through winter in an urban temperate climate. Specifically, we investigated the influence of ground cover in low tunnels at 10 urban garden sites and in low tunnels within a high tunnel at the University of the District of Columbia's Farm to ascertain the influence on the yield and nutrients of Red Russian kale, soil temperature, air temperature, weed pressure, and abundance of aphids.

Fig. 1 - Example layout for growing Red Russian kale at 10 urban garden sites in the USA. Kale is planted in a low tunnel with bare soil in the foreground, black plastic mulch in the middle ground, and straw mulch in the background.

2. Materials and Methods

Sites and production systems

Urban garden sites. We started kale seedlings on 25 September 2020 in a high tunnel at the University of the District of Columbia's 58 ha Firebird Farm (Beltsville, MD, USA) before transplanting them into 10 urban garden sites in Virginia, Maryland, and Washington, DC between 21 October through 4 November 2020. Each urban site contained three plots which were weeded and randomly assigned a ground cover treatment: bare soil, black plastic mulch, or straw mulch (Fig. 1). Each plot was approximately 3 m x 0.6 m with 8-9 kale plants spaced 0.3 m apart. We used 1.0 mil thick black plastic mulch (Dubois Agrinovation, Ontario, Canada) and applied straw to a depth of 7.6 cm. We constructed low tunnels over each plot using wire hoops (0.9 m high and 0.3 m wide) and 0.8 mil low tunnel clear plastic (Agriculture Solutions, Kingfield, ME, USA), the edges of which were secured with pins and weights. Ground cover and low tunnels were installed the same day as planting at all locations except one where the initial planting failed. Warm ambient temperatures initially required the low tunnels to be left open until 16 November 2020; at this point they were closed for the remainder of the research.

High tunnel. Red Russian kale from the same batch of seedlings used for the garden sites was transplanted 28 October 2020 into a high tunnel (30.48 m long x 9.15 m wide x 4.57 m high) covered with a 6-mil thick double-layered polyethylene film (Sun Master®, Farmtek, Dyersville, IA, USA) and located at the Firebird Farm (Fig. 2). We established four plots, each of which was 3 m by 0.9 m, in each of four parallel rows (i.e., blocks) and used a randomized complete block design to assign one of four treatments to each plot. The treatments were: 1) bare soil; 2) bare soil inside a low tunnel, 3) black plastic mulch inside a low tunnel; and 4) straw mulch inside a low tunnel. Ground cover and low tunnel treatments were set up 20 October 2020 as previously described for the urban garden sites. Each plot contained nine kale plants, spaced 0.3 meters apart. Warm ambient temperatures initially required the low tunnels to be left open until 3 December 2020, at which point they were closed until 8 March 2021.

Fig. 2 - Red Russian kale in a high tunnel in Maryland, USA. Kale was planted in one of four treatments: bare soil (no ground cover and low tunnel); bare soil + low tunnel (no ground cover and covered with a low tunnel); black plastic + low tunnel (black plastic mulch and covered with a low tunnel); straw + low tunnel (straw mulch and covered with a low tunnel). Sixteen experimental plots are arranged in four rows, with a row of non-experimental plants separating the rows into pairs.

Environmental data and plant yield

We measured air temperatures hourly in all plots at garden sites and in the high tunnel using HOBO® Pendant MX 2201 Data Loggers (Onset, Bourne, MA, USA). They were initially placed flat and 7.62 cm above the ground upon planting kale. If plants in any plot grew large enough to cover the data logger, all sensors at that site were then raised to 33 cm (garden sites) or 40.6 cm (high tunnel) above the ground. We also measured soil temperature in each plot hourly using HOBO® Pendant MX 2201 Data Loggers or, in two cases, a HOBO MX2303 Two External Temperature Sensors Data Logger (Onset, Bourne, MA, USA). Data loggers were buried 7.62 cm below the surface of the soil upon planting. Data from all loggers were downloaded weekly.

We began collecting weed mass biweekly starting 26 November 2020. Weed mass was measured by removing all weeds from a plot, shaking dirt from the roots, and weighing the total fresh mass. We scouted for all insect pests bi-weekly from 10 December 2020 to 17 March 2021, but aphids were the only pest regularly present. To measure the abundance of aphids, we removed the leaf with the most aphids from each plant that had more than five aphids present, stored them in a freezer, and then counted the aphids under a dissecting microscope. After leaves were removed, we treated all plants at a site using 59 ml Triple Action Neem Oil (Southern Agricultural Insecticides, Inc., Boone, NC, USA) per 3.8 l water to reduce pest infestations.

Biweekly harvest began on or after 10 December 2020 when at least three plants per plot had one or more leaf blades 20.3 cm long. Individual leaves 20.3 cm or longer were cut 5 to 7.6 cm from the stalk of the plant and all fresh mass from a plot was weighed. Final harvest was 17 March 2021.

Nutrient analysis

Due to low yield of kale in garden sites, nutrient analysis could only be conducted with samples from the high tunnel. Approximately 12 fully developed leaves from each of three subsamples within a treatment were shipped on ice to Waypoint Analytical (Leola, PA) to quantify mineral content, including calcium (%), magnesium (%), nitrogen (%), phosphorous (%), potassium (%), sodium (%), sulfur (%), boron (ppm), copper (ppm), iron (ppm), manganese (ppm), and zinc (ppm) using the United States Environmental Protection Agency (US EPA) SW-846 method and American Oil Chemists' Society (AOCS) Official Method Ba 4e-93 for nitrogen. Amounts are reported on a dry matter basis. Additionally, we noticed a distinct difference in texture between kale plants in bare soil with no low tunnel versus all other treatments which had a low tunnel, which we suspected was due to fiber content. So, we harvested approximately 100 g of fresh kale leaves per subsample in the two bare soil treatments (with and without a low tunnel) and mailed them on ice to Eurofins Microbiology Laboratories (Lancaster, PA). Eurofins Microbiology Laboratories analyzed total dietary fiber using the Association of Official Analytical Chemists (AOAC) method 991.43. The percent content of fiber is reported on a fresh matter basis. Leaves harvested for all nutrient analysis were included in our measurements of total yield.

Data analysis

In all analyses, the plot is the replicate and the individual kale plants are subsamples. We used the mean yield per plant, plot, and month in a mixed model, (PROC GLIMMIX; SAS Institute, 2020) to determine whether yield differed among the three ground cover treatments at garden sites, with mean air and soil temperatures as covariates, treatment as a fixed effect, and site as a random effect. We also used separate mixed models (PROC GLIMMIX; SAS Institute, 2020) to determine whether weed mass and the number of aphids per plant differed among

the three treatments at garden sites, with treatment as a fixed effect and site as a random effect.

We used the mean yield per plant, plot, and month in a mixed model, (PROC GLIMMIX; SAS Institute, 2020) to determine whether yield differed among the four ground cover treatments in the high tunnel experiment, with mean air and soil temperature as covariates and treatment as a fixed effect. Differences in weeds and most nutrients, except fiber, across treatments in the high tunnel experiment were also analyzed with separate general linear models (PROC GLM; SAS Institute, 2020). We used a negative binomial model (PROC GENMOD; SAS Institute, 2020) to determine whether the total number of aphids differed among the four treatments. This analysis differs from the aphids at garden sites because the number of plants in each plot was variable, but the number of plants in each plot in the high tunnel was the same, allowing for a count model to be used. Differences in dietary fiber between bare soil plots with and without a low tunnel were compared with a t-test. We used base-10 log transformations prior to some analyses to meet assumptions of normality but means for non-transformed data are presented in the results. We used means separation tests for all analyses to determine which means differed ($P<0.05$).

3. Results

Garden sites

The amount of kale harvested from garden sites was extremely low and did not differ across ground cover treatments ($F_{2,106} = 0.24$, $P = 0.78$) (Table 1). However, yield was higher when mean air ($F_{1,106} = 20.1$, $P<0.01$) and soil temperature $F_{1,106} = 16.6$, $P<0.01$) were higher. The mean marketable yield/plant/treatment generally decreased over time from a maximum of 37.5 g in December to 11.4 g, 3.7 g, and 4.0 g in January, February, and March, respectively. Ground cover treatments influenced weed mass, with the greatest mass manually removed from plots with bare ground, a lesser amount from plots with straw, and nearly none from plots with black plastic mulch ($F_{2,18} = 58.1$, $P < 0.01$) (Table 1). The number of aphids per plant did not vary across treatments ($F_{2,18} = 0.95$, $P = 0.40$) (Table 1).

Table 1 - Mean yield per plant of Red Russian kale, mass of weeds, and number of aphids per plant across three treatments at 10 urban garden sites in Virginia, Maryland, and Washington, DC from October 2020 to March 2021. The sample size for each measurement equals the number of garden sites (10)

Variable	Ground cover treatment		
	Bare soil	Black plastic	Straw mulch
Crop yield (g)	14.1 (30.9)	9.8 (25.6)	5.1 (16.6)
Weeds (g)	313 (349) a	68.6 (59) b	7.0 (8.8) c
No. aphids per plant	48.4 (72.2)	19.7 (20.5)	23.0 (49.1)

Each urban site included three plots with a ground cover treatment: bare soil, black plastic mulch, or straw mulch. Means with different letters within rows are different ($P<0.05$). Standard deviations are provided in parentheses.

High tunnel

Kale yield varied across treatments ($F_{3,58} = 4.1$, $P = 0.01$), with the greatest yield from plots with straw and plastic mulch and the least yield from plots with bare soil and no low tunnel (Table 2). Yield was also greater when mean air ($F_{1,58} = 20.2$, $P < 0.01$) and soil temperature ($F_{1,58} = 31.9$, $P < 0.01$) were higher, and this was influenced by treatments. Specifically, mean air temperature was 19.1°C in plots with straw

Table 2 - Mean yield per plant of Red Russian kale, mass of weeds, and total number of aphids across four treatments in a high tunnel in Maryland from October 2020 to March 2021. There were four plots per treatment

Variable	Treatment			
	Bare soil	Bare soil + low tunnel	Black plastic + low tunnel	Straw + low tunnel
Crop yield (g)	917 (830) b	1334 (632) ab	1384 (709) a	1390 (577) a
Weeds (g)	141 (189) b	142 (96) b	2.8 (4.3) b	65 (110) ab
No. aphids	231 (285)	118 (141)	66 (90)	65 (121)

Each plots within the high tunnel was assigned one of four treatments: bare soil (no ground cover and low tunnel); bare soil + low tunnel (no ground cover and covered with a low tunnel); black plastic + low tunnel (black plastic mulch and covered with a low tunnel); straw + low tunnel (straw mulch and covered with a low tunnel). Means with different letters within rows are different ($P<0.05$). Standard deviations are provided in parentheses.

mulch, 18.5°C in plots with plastic mulch, and only 15.6°C in plots with bare soil and no low tunnel. Mean soil temperature was 12.2°C in plots with straw mulch, 12.5°C in plots with plastic mulch, and only 10.9°C in plots with bare soil and no low tunnel.

Ground cover treatments influenced weed mass with the greatest mass manually removed from plots with bare soil (with or without a low tunnel) and nearly none from plots with black plastic mulch ($F_{3,12}$ = 7.6, P<0.01). However, the total number of aphids did not differ across treatments (F_3 = 1.75, P = 0.63) (Table 2).

Analysis of 12 plant elements in the high tunnel experiment revealed that B, Ca, Fe, K, Mg, Mn, N, Na, P, and Zn showed no variation across treatments (all P-values > 0.07). However, Cu was highest in plants in straw mulch and lowest in those grown in bare soil without a low tunnel (Table 3). Plants in all treatments with a low tunnel exhibited higher S compared to those without a low tunnel (Table 3). Furthermore, plants grown in bare soil under a low tunnel displayed greater dietary fiber compared to those in bare soil without a low tunnel (Table 3).

4. Discussion and Conclusions

One potential benefit of urban agriculture is the possibility of producing local, nutritious foods year-round, such as Red Russian kale. However, winter production in unheated structures such as low and high tunnels in temperate environments relies on capturing sufficient solar radiation to support crop production. Heat generated within these structures during daylight will dissipate from those structures at night. Heat storage and the rate of dissipation are influenced by environmental conditions outside of the tunnel and the size and construction of the protected structures. For example, secondary covers within a high tunnel may increase the amount of time plants experience optimal growing temperatures during colder months and modulate extreme temperatures (Borrelli *et al.*, 2013; Drost *et al.*, 2017). Small tunnels with a single layer of plastic will experience faster heat loss than larger tunnels with multiple layers of plastic. Insulating ground covers may act to slow down heat loss below- and above-ground (Bhardwaj, 2013). In our study straw and black plastic ground covers combined with low tunnels were not sufficient to produce a viable yield of Red Russian kale at 10 garden sites. Yield at these garden sites was higher when air temperature and soil temperature were higher, but these temperatures were likely influenced by geographic location because the ground covers were not associated with differential yield. Outside temperature around the low tunnels was likely too cold, and the area within the tunnels too small, to increase or retain sufficient heat for crop production. However, cultivating kale in a high tunnel resulted in good yields, especially when paired with a low tunnel and either plastic or straw mulch; this combination led to the highest air and soil temperatures. Our results should not be taken to mean that low tunnels alone cannot be used to produce kale or that a high tunnel is necessary. These systems may vary in their effectiveness under other environmental conditions or if made from other materials. Also, other varieties of kale may perform better in these systems than Red Russian kale because different varieties, cultivars, and genotypes of a plant species respond differently to cultivation methods and environmental conditions (Yoder and Davis, 2020; Richardson and Arlotta, 2021, 2022; Richardson *et al.*, 2022).

Mineral absorption in plants is known to be influenced directly or indirectly by air and soil tempera-

Table 3 - Mean content of nutrients that differed in Red Russian kale across four treatments in a high tunnel in Maryland from October 2020 to March 2021. There were four plots per treatment

Nutrient	Treatment				P
	Bare soil	Bare soil + low tunnel	Black plastic + low tunnel	Straw + low tunnel	
Copper (ppm)	9.8 b	11.4 ab	10.7 ab	11.7 a	0.02
Sulfur (%)	0.88 b	1.24 a	1.23 a	1.16 a	<0.01
Dietary fiber (%)	4.4 a	3.4 b	NA	NA	<0.01

Each plot within the high tunnel was assigned one of four treatments: bare soil (no ground cover and low tunnel); bare soil + low tunnel (no ground cover and covered with a low tunnel); black plastic + low tunnel (black plastic mulch and covered with a low tunnel); straw + low tunnel (straw mulch and covered with a low tunnel). Means with different letters within rows are different (p<0.05).

ture (Tachibana, 1982; Inthichack *et al.*, 2013). Given this understanding, we expected to observe variation in mineral content among the different treatments. However, our results indicate that the use of low tunnels and ground covers in the high tunnel did not influence mineral content, except for copper and sulfur, which were lower in plots without a low tunnel or ground cover than some other treatments. Although yield and mineral content, to an extent, were improved with low tunnels and ground cover in the high tunnel, dietary fiber was higher when no low tunnel or ground cover was used. This may help urban farmers grow kale for instances in which maximizing dietary fiber is a priority. However, it is important to consider that these plants were also much smaller in structure and yielded less, which could potentially affect profitability.

Beyond the influence on the Red Russian kale, the ground covers also had an impact on weed growth. As expected, the use of plastic and straw mulch suppressed weed growth. Contrary to our expectations, we did not find evidence that these ground covers in combination with low tunnels increased the abundance of insect pests. Insect pests were observed in all treatments in garden sites and the high tunnel. It is likely the presence of insect pests in all treatments can be attributed to the warmer air temperature fostered by the low and high tunnels alone, rather than the addition of ground covers.

In conclusion, our work demonstrates that Red Russian kale, a highly nutritious, desired, and appropriate crop for urban systems can be grown in temperate regions throughout the winter using specific combinations of tunnels and ground covers. These findings open opportunities for year-round cultivation of this crop in urban systems. Future research could continue to build upon our findings to elucidate the best methods that maximize yield and nutrients while not significantly contributing to increased abundance of insect pests.

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Pumpkins (*Cucurbita* spp.) diversity and their associated microbiota

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Abstract: Root-associated microbiota play a key role in plant growth, resilience, and health. In this study, the microbial community structure in the rhizosphere of 12 pumpkins accessions belonging to three *Cucurbita* species i.e. *C. pepo*, *C. maxima*, and *C. moschata*, was monitored using the soil dilution plating technique on specific media. All accessions tested were also screened for their production and yield parameters. Based on Principal Component Analysis (PCA), 4 accessions of *C. maxima* (namely C5, C23, C14.2 and C6.2) were characterized by the greatest average fruit weight and yield, the highest actinomycetes, bacterial, *Trichoderma* spp. and *Aspergillus* spp. communities, and the lowest total fungal population in their rhizosphere. Positive correlations were noted between fruit fresh weight, culturable bacteria and *Trichoderma* spp. populations in the rhizopshere of pumpkins accessions. Negative correlations were noted between fruit weight and yield parameters and the total culturable fungal populations. The current study clearly demonstrated that the rhizosphere soil microbial communities have been shaped by *Cucurbita* species and accessions. Based on the significant links observed between soil microbiota and yield parameters, future pumpkin breeding programs could be focused on the selection of accessions that are quite able to exploit these associated beneficial microbial communities.

1. Introduction

Plants through their root system and surrounding soil influenced by root exudates represent an interesting ecological niche for the development of soil microbiota which are able to colonize the rhizosphere, roots and eventually move to the above-ground plant parts (Compant *et al*., 2019). Due to its ecological importance and functional diversity, the rhizosphere microbiome was intensively explored for various features (Marques *et al*., 2014; Edwards *et al*., 2015; Gopal and Gupta, 2016; Compant *et al*., 2019). Microbiotas associated to roots are derived from the soil environment which contains highly diverse microorganisms including Acidobacteria, Verrucomicrobia, Bacteroidetes, Proteobacteria,

Planctomycetes, and Actinobacteria (Fierer, 2017). Seeds may be colonized by various microorganisms which proliferate later in the roots of the developing plant and colonize the rhizosphere (Compant *et al*., 2019).

Soil microbial communities play key roles in plant development and health (Philippot *et al*., 2013; Adam *et al*., 2018). In fact, they may be associated to growth promotion, improved nutrient uptake, and enhanced tolerance to various abiotic and/or biotic stresses (Trivedi *et al*., 2020). The below-ground microbial composition is influenced by many abiotic and biotic factors including soil traits (pH, salinity, structure, moisture, organic matter, environmental conditions), relative abundance of soilborne bio-aggressors, plant species, genotypes, and agricultural and disease management practices (Hardoim *et al*., 2015; Fierer, 2017; Compant *et al*., 2019). All the above-mentioned factors contribute, at variable degrees, to the definition of the root microbial community structure together with the host-related factors like plant age and developmental stage, health status and the composition of root exudates (Bulgarelli *et al*., 2012). Based on Carelli *et al*. (2000) investigation, the rhizosphere community composition varies between plant species and even within the same species between plant genotypes. Also, the root exudates play a key role in recruiting and shaping the soil microbial population structure as they serve as nutrient sources for rhizosphere microorganisms (Sung *et al*., 2006) and represents an important component of communication with rhizosphere-inhabiting microorganisms (Haichar *et al*., 2014). Hence, the variation in the chemical composition of root exudates between and within plant species (Grayer *et al*., 2004) may lead to the development and the proliferation of a phylogenetically diverse array of microorganisms. The chemical composition of root exudates, resulting of different below-ground interactions and factors (soil chemical and physical properties, plant species, age, etc), may impact the soil microbial community structure and function by influencing plant physiology and development (Griffiths *et al*., 1999). In fact, among the members of the rhizosphere microbiome, some are beneficial for plant growth and resilience but others may be phytopathogenic exhibiting capacity to overcome the innate plant defense system and to cause devastating diseases (de Faria *et al*., 2021).

Pumpkin (*Cucurbita* spp.) is an extraordinary vegetable species that may be exploited for medicinal and nutritional features (Tlili *et al*., 2020; Hosen *et al*., 2021; Chikh-Rouhou *et al*. 2023 b). However, pumpkin cultivation is still ignored in some countries. *Cucurbita* pepo L., *C. maxima* Duchesne, and *C. moschata* Duchesne are three pumpkin species economically important which are grown over various agricultural regions worldwide (Maynard *et al*., 2002). In Tunisia, pumpkin has significant economic importance especially as familiar agriculture because of its rusticity, high nutritional values and long post-harvesting conservation. There is no improved cultivar in Tunisia and the production of *Cucurbita* is based on local accessions and landraces. Chikh-Rouhou *et al*. (2019, 2023 a, 2023 b) evidenced that pumpkin landraces collected from farmers of the Centre-East of Tunisia belongs to three species namely *C. maxima*, *C. pepo*, and *C. moschata* with a predominance of *C. maxima*. Pumpkins face a number of constraints including a shortage of genetically improved seeds, infections with various pests and pathogens (Ndinya, 2019) in addition to the plant parasitic nematode *Pratylenchus* (Zhao *et al*., 2022). Developing new cultivars with superior qualities, higher mineral contents, important yield and average weight of fruits, potential resistance towards pests and fungal diseases, tolerance to environmental difficulties, shelf lives enhancement is highly required (Paris, 2016; Seymen *et al*., 2016; Hosen *et al*., 2021).

Breeding plants for beneficial plant-microbe interactions is an emerging field mainly focusing the below-ground interactions in the rhizosphere and their valorization for the development of economically and ecologically interesting plant material (Bakker *et al*., 2012; Adam *et al*., 2018). In fact, breeding shapes the composition of the root-associated microbial communities including the antagonistic potential towards the encountered pathogens (Peiffer and Ley, 2013; Bouffaud *et al*., 2014; Cardinale *et al*., 2015). Thus, breeding strategy is recently focused on genotypes-microbial holobiont interactions in order to generate diverse new phenotypes without altering plant genomic information (Wei and Jousset, 2017; Adam *et al*., 2018; Wille *et al*., 2018).

Therefore, this study aimed to select the most productive pumpkin accession among 12 tested, to determine their associated culturable soil microbial community and to search for an eventual link between fruit and yield parameters and their associated microorganisms and soil traits.

2. Materials and Methods

Plant material

Twelve (12) pumpkin (*Cucurbita* spp.) accessions belonging to three *Cucurbita* species (*C. maxima*, *C. moschata*, and *C. pepo*) are used in this study. Their main traits are detailed in Table 1 and figure 1. They were obtained from the Cucurbits breeding program at the Regional Research Centre on Horticulture and Organic Agriculture (CRRHAB), Chott-Mariem, Tunisia.

For each accession, seeds were sown in cell trays and maintained at 25°C under greenhouse conditions. At the two-true-leaf growth stage, they were further transplanted (end of March) to an open field at the experimental station of CRRHAB of Sahline, Tunisia (N35° 45'05'', E10°42'39'').

Experimental design

Pumpkins seedlings were transplanted into rows with a distance of 120 cm between seedlings within the same row and 80 cm between rows. The trial was conducted under drip irrigation system without inputs. Cattle manure was applied at a rate of 500 Kg ha^{-1} before planting. The experimental design was a completely randomized block design. Two replicates of six seedlings each were used per each accession tested.

Soil sampling

Composite soil samples from each replicate were collected at the initial state (before planting) (Table 2) and four times post planting i.e. at 30, 60, 90, 150 days post-planting (DPP).

After planting, three soil cores (7 cm in diameter ×

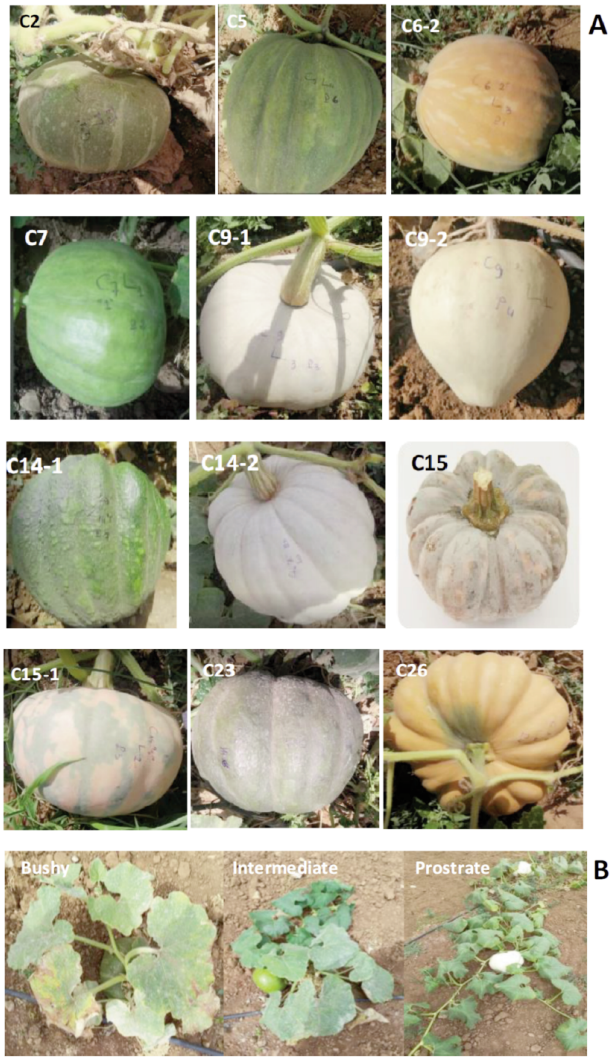

Fig. 1 - Diversity of Pumpkin (*Cucurbita* spp.) accessions used in the study (A) and their plant growth habits (B). C7, C15: *Cucurbita pepo*. C2, C5, C6.2, C9.1, C9.2, C14.2, C15.1, C23: *Cucurbita maxima*. C14.1, C26: *Cucurbita moschata.*

Table 1 - Pumpkin accessions (species and characteristics) used in this study and their main traits

Accession codes	*Cucurbita* species	Plant growth habit	Fruit shape	Flesh color
C2	*C. maxima*	Bushy	Transverse broad elliptic	Yellow
C5	*C. maxima*	Prostrate	Medium elliptic	Orange
C6.2	*C. maxima*	Intermediate	Globular	Yellowish orange
C7	*C. pepo*	Bushy	Globular	Cream
C9.1*	*C. maxima*	Prostrate	Transverse medium elliptic	Yellow
C9.2	*C. maxima*	Intermediate	Heart shaped	Yellowish orange
C14.1	*C. moschata*	Intermediate	Top shaped	Yellowish orange
C14.2	*C. maxima*	Prostrate	Medium elliptic	Yellow
C15*	*C. pepo*	Bushy	Transverse elliptical	Yellow
C15.1*	*C. maxima*	Intermediate	Transverse medium elliptic	Orange
C23	*C. maxima*	Prostrate	Transverse elliptical	Orange
C26	*C. moschata*	Prostrate	Transverse broad elliptic	Orange

* Highly susceptible to powdery mildew (data not shown).

Table 2 - Soil characteristics estimated at the initial state (before pumpkin planting) as determined by soil dilution [z] plating on selective media

Soil characteristic	Data
Initial soil characteristics	
pH	7.42
EC (dS m^{-1})	0.56
Culturable microbial population (CFU [y] g^{-1} fresh	
Total bacteria (× 10^7)	2.99
Actinomycetes (× 10^4)	0.95
Total fungi (× 10^4)	1.62
Aspergillus spp. (× 10^3)	0.12
Trichoderma spp. (× 10^3)	1.12
Fusarium spp. (× 10^3)	0.18

[z] Soil sample was a composite soil from twenty soil cores collected before planting and soil dilution was made from a concentration of 10% (w v^{-1}).
[y] CFU= Colony-Forming Unit.

15 cm in depth) were removed from the rhizosphere soil of each sampled plant and were combined to make one composite soil per accession. At the initial state (before planting), ten soil cores were removed and were combined to make one composite soil sample. Two replicates were considered for each soil sampling.

Once brought to laboratory, soil samples were passed through a 2-mm sieve to remove rocks and large organic debris. They were stored in plastic bags at 10°C until use. Two subsamples were processed from each soil sample.

Determination of soil pH and electrical conductivity (EC)

Each composite soil sample was air-dried and suspended into distilled water (1:10 soil H_2O^{-1} ratio). Soil filtrates obtained by filtration through Whatman paper No. 1 were analyzed for the determination of their pH and electrical conductivity (EC) using a glass electrode (VWR sympHony®) and a digital conductivity meter (HANNA®), respectively.

Estimation of soil microbial community structure

General populations of culturable soil microorganisms were determined using the soil dilution plating techniques on various agar media according to Larkin and Honeycutt (2006) with some modifications. For each subsample taken from each composite soil, 10 g were added to 90 ml of sterile 0.2% water agar, vigorously stirred for 30 min, serially diluted and a-100 µl sample was plated on 10% Tryptic Soy Agar (TSA) for total bacterial counts, Yeast Malt Agar (ISP medium No. 2) amended with 75 mg l^{-1} of nalidixic acid and 100 mg l^{-1} of cyclohexamide for actinomycete counts, and Potato Dextrose Agar (PDA) amended with 300 mg l^{-1} of streptomycin sulphate for total fungal counts. Three replicates of one plate each were used for each soil subsample.

Bacterial and actinomycete plates were incubated at 28°C for 2 and 14 days, respectively, and fungal plates were maintained at 25°C for 7 days. Colonies of *Trichoderma* spp., *Aspergillus* spp., and *Fusarium* spp. were identified based on their macro- and micro-morphological traits (Barnett and Hunter, 1987) under light microscope and counted separately. Colony-forming units (CFU) were counted to estimate the microbial density on each selective medium (Marin *et al*., 2013). The soil microbial population counts were estimated per 1 g of fresh soil.

Yield parameters

The average fruit weight and the average yield per plant were noted at five months post-planting. The average fruit weight parameter was determined for three randomly sampled plants.

Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software for Windows version 16.0. Data for pH, EC of soil samples and rhizosphere microbial population counts were analyzed according to a completely randomized factorial model with two factors (Accessions tested × Sampling times). As for yield parameters, data were analyzed according to a completely randomized block design. Experiments were repeated twice. Means were separated using Tukey test to identify significant pair-wise differences at $P \leq 0.05$.

Correlations between fruit weight and yield parameters and soil characteristics (pH, EC and microbial community structure) were carried out using Pearson's test at $P \leq 0.05$.

For an overview of pumpkins accessions distribution, and to explore soil microbial community contributing to classification, a Principal Component Analysis (PCA) was also performed using SPSS.

3. Results

Variation of soil pH and EC

ANOVA analysis of pH values varied significantly

(at $P \leq 0.05$) depending on sampling times only. No significant difference was noted between pumpkins accessions and between both factors (Table 3). A significant decrease of pH values of about 9.9 to 12.7% was noted at harvest (150 DPP) as compared to soil samples collected at 30, 60 and 90 DPP (Table 3).

Table 3 - pH and electrical conductivity (EC) of soil samples removed from the rhizosphere of pumpkins depending on accessions tested and sampling times

Soil samples	pH	EC (dS m^{-1})
Accessions [z] means [y]		
C2	7.30 a	0.507 ef
C5	7.22 a	0.45 f
C6.2	7.27 a	0.61 bc
C7	7.18 a	0.63 abc
C9.1	7.24 a	0.69 a
C9.2	7.24 a	0.508 def
C14.1	7.17 a	0.57 cd
C14.2	7.21 a	0.66 ab
C15	7.14 a	0.62 abc
C15.1	6.69 a	0.56 cde
C23	7.19 a	0.47 f
C26	7.27 a	0.503 ef
Sampling times means [x]		
30 DPP [w]	7.44 a	0.62 a
60 DPP	7.25 a	0.61 ab
90 DPP	7.48 a	0.4 c
150 DPP	6.53 b	0.5 b
Source of variation	p-values	
Accessions (Acc)	0.27	P ≤ 0.001
Sampling times (ST)	P≤0.001	P ≤ 0.001
Acc × ST	0.48	P ≤ 0.001

[z] C7 and C15= *Cucurbita pepo*. C2, C5, C6.2, C9.1, C9.2, C14.2, C15.1 and C23= *C. maxima.* C14.1 and C26= *C. moschata*.
[y] Accessions means (for all sampling times combined) followed by the same letter are not significantly different according to Tukey test at P≤0.05.
[x] Sampling times means (for all accessions combined) followed by the same letter are not significantly different according to Tukey test at P≤0.05.
[w] DPP= Days post-planting.

ANOVA analyses revealed a significant variation in EC values among accessions, sampling times and their interaction (Table 3). The highest EC values were recorded in the rhizopshere of *C. maxima* C9.1, *C. maxima* C14.2, *C. pepo* C7 and *C. pepo* C15. As for the sampling time effect on this parameter, the EC of the rhizosphere soil associated to the twelve pumpkins accessions was 34.4-35% and 18.1-19% higher at 30-60 DPP than at 90 and 150 DPP, respectively (Table 3).

Variation of the culturable soil microbial structure

The number of bacterial and actinomycetes colonies varied significantly (at $P \leq 0.05$) among pumpkins accessions, sampling times and their interaction (Table 4). The highest population of culturable bacteria was obtained from the rhizosphere of *C. maxima* C23 and *C. pepo* C15 which was 33.1-55.8% and 15.8-44.4% more abundant than those of the remaining accessions (Table 4). The abundance of culturable bacteria in the rhizosphere of all the remaining accessions was significantly comparable. Concerning the effect of the sampling times (all accessions combined) on this parameter, bacterial colonies counts

Table 4 - Culturable bacterial, actinomycetes and fungal population densities in soil samples (CFU g^{-1} of fresh soil) removed from the rhizosphere of pumpkins plants depending on accessions tested and sampling times

Culturable microbiome population	Bacteria	Actinomycetes	Fungi
Accessions [z] means [y]			
CFU [x] g^{-1} of fresh soil	× 10^8	× 105	× 105
C2	1.68 bc	0.96 bc	1.36 a
C5	1.62 bc	1.68 a	0.93 a
C6.2	2.24 bc	1.20 abc	1.22 a
C7	1.48 c	1.08 bc	1.69 a
C9.1	1.59 bc	1.08 bc	1.59 a
C9.2	1.80 bc	0.96 bc	1.58 a
C14.1	1.77 bc	1.05 bc	1.63 a
C14.2	2.04 bc	1.13 abc	1.32 a
C15	2.66 ab	0.82 c	1.45 a
C15.1	1.98 bc	0.90 c	1.28 a
C23	3.35 a	1.53 ab	1.02 a
C26	1.93 bc	0.74 c	1.20 a
Sampling times means [w]			
CFU g^{-1} of fresh soil	× 108	× 105	× 105
30 DPP [v]	3.15 a	0.93 b	1.57 ab
60 DPP	3.08 a	2.86 a	1.86 a
90 DPP	1.06 b	0.27 c	1.41 b
150 DPP	0.74 b	0.32 c	0.58 c
Sources of variation		p-values	
Accessions (Acc)	P≤0.001	P≤0.001	0.06
Sampling times (ST)	P≤0.001	P≤0.001	P≤0.001
Acc × ST	P≤0.001	P≤0.001	0.15

[z] C7 and C15= *Cucurbita pepo*. C2, C5, C6.2, C9.1, C9.2, C14.2, C15.1 and C23= *C. maxima.* C14.1 and C26= *C. moschata*.
[y] Accessions means (for all sampling times combined) followed by the same letter are not significantly different according to Tukey test at P≤0.05.
[x] CFU= Colony forming unit.
[w] Sampling times means (for all accessions combined) followed by the same letter are not significantly different according to Tukey test at P≤0.05.
[v] DPP= Days post-planting.

from the rhizosphere of all pumpkins accessions noted at 30 and 60 DPP were 65.6-66.3 and 75.9-76.5% significantly higher than those recorded at 90 and 150 DPP, respectively.

Actinomycetes community was abundant on the rhizopshere of *C. maxima* C5, *C. maxima* C6.2, *C. maxima* C14.2 and *C. maxima* C23 which was 35.7-55.9%, 10-38-3%, 4.4-34.5% and 29.4-51.6% higher than that associated to the remaining accessions. For all pumpkins accessions combined, the actinomycetes population was 67.5, 90.5 and 91.9% significantly higher at 60 DPP than at 30, 90 and 150 DPP, respectively.

Data given in Table 4 showed that the total culturable fungal community varied significantly (at $P \leq 0.05$) depending on sampling times only and that all accessions tested exhibited significantly comparable fungal community populations. Fungal colonies recovered from the rhizosphere of all pumpkins accessions at 60 DPP were 15.6, 24.2 and 68.8% significantly higher than those recovered at 30, 90 and 150 DPP, respectively.

As for fungal community structure, culturable *Aspergillus* spp. and *Trichoderma* spp. populations varied significantly (at $P \leq 0.05$) in the rhizosphere of pumpkins plants depending on tested accessions, sampling times and their interaction (Table 5). For instance, the rhizospheric *Aspergillus* spp. community associated to *C. maxima* C14.2 was significantly 40-48.8% more abundant than that associated to *C. moschata* C26 and *C. maxima* C2 accessions. Furthermore, *Trichoderma* spp. population was significantly 75.9-84.1% higher at the rhizosphere of *C. maxima* C14.2 than at that of *C. pepeo* C7 and *C.*

Table 5 - Culturable fungal population structure in soil samples (CFU g^{-1} of fresh soil) removed from the rhizosphere of pumpkins plants depending on accessions tested and sampling times

Culturable fungal population	*Aspergillus* spp.	*Trichoderma* spp.	*Fusarium* spp.
Accessions [z] means [y]			
CFU [x] g^{-1} of fresh soil	$\times 10^4$	$\times 10^4$	$\times 10^4$
C2	1.92 b	1.33 abc	0.07 a
C5	3 ab	2 ab	0.83 a
C6.2	3.08 ab	0.66 abc	1.5 a
C7	3.33 ab	0.50 bc	0.07 a
C9.1	3.25 ab	0.33 c	0.83 a
C9.2	3 ab	0.83 abc	1.66 a
C14.1	2.42 ab	1.50 abc	0.08 a
C14.2	3.75 a	2.08 a	0.09 a
C15	2.58 ab	1.58 abc	0.08 a
C15.1	2.83 ab	1.17 abc	0.07 a
C23	2.75 ab	1.75 abc	0.09 a
C26	2.25 b	1.17 abc	0.08 a
Sampling times means [w]			
CFU g^{-1} of fresh soil	$\times 10^4$	$\times 10^4$	$\times 10^4$
30 DPP [v]	0.83 c	1.08 ab	0.27 a
60 DPP	0.55 c	0.72 b	0.08 a
90 DPP	1.44 b	1.58 a	1.38 a
150 DPP	9.81 a	1.58 a	0.27 a
Sources of variation		p-values	
Accessions (Acc)	$P \leq 0.01$	$P \leq 0.01$	0.14
Sampling times (ST)	$P \leq 0.001$	$P \leq 0.01$	0.68
Acc × ST	$P \leq 0.001$	$P \leq 0.001$	0.35

+[z] C7 and C15= *Cucurbita pepo*. C2, C5, C6.2, C9.1, C9.2, C14.2, C15.1 and C23= *C. maxima.* C14.1 and C26= *C. moschata*.
[y] Accessions means (for all sampling times combined) followed by the same letter are not significantly different according to Tukey test at $P \leq 0.05$.
[x] CFU= Colony forming unit.
[w] Sampling times means (for all accessions combined) followed by the same letter are not significantly different according to Tukey test at $P \leq 0.05$.
[v] DPP= Days post-planting.

maxima C9.1 (Table 5).

Aspergillus spp. colonies recovered from the rhizosphere of all pumpkins accessions at 150 DPP were 85.3 and 94.4% significantly higher than those recovered at 90 and 30-60 DPP, respectively. *Trichoderma* spp. population estimated was significantly higher (+54.4%) at 150 and 90 DPP than at 60 DPP. Concerning *Fusarium* spp. populations, no significant differences were detected between pumpkins accessions and sampling times nor their interaction (Table 5).

Variation of fruit production and yield among pumpkins accessions tested

Analysis of variance revealed a significant (at $P \leq 0.05$) variation of the average fruit weight between the pumpkin accessions. The highest average fruit weights ranging between 4.18 and 8.48 Kg were noted in the accessions C5, C14.2, C15.1 and C23 of *C. maxima* and C14.1 of *C. moschata* whereas for the remaining seven pumpkins accessions, this parameter varied between 2.53 and 3.9 Kg (Fig. 2A).

The average fruit yield produced per plant varied significantly (at $P \leq 0.05$) among pumpkins accessions. Four *C. maxima* accessions (namely C5, C6.2, C14.2, and C23) and one *C. moschata* accession (C26) produced significantly the highest fruit yields per plant (5.31-6.21 Kg plant^{-1}) than the remaining ones (0.91-3.96 Kg plant^{-1}) (Fig. 2B).

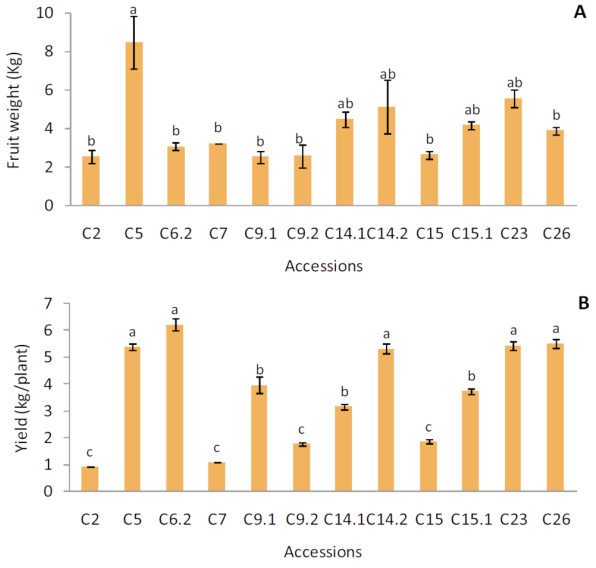

Fig. 2 - A, B Average fruit weight and yield per plant of pumpkins accessions noted five months post-planting. Bars sharing the same letter are not significantly different according to Tukey test at $P \leq 0.05$. The average fruit weight (A) and the average yield (B) per plant were determined at harvest. C7 and C15: *Cucurbita pepo*. C2, C5, C6.2, C9.1, C9.2, C14.2, C15.1, and C23: *C. maxima.* C14.1 and C26: *C. moschata.*

Correlation between production and yield parameters and soil characteristics

Pearson's correlation analysis indicated that the average fruit weight was significantly and positively correlated to the associated actinomycetes community (r= 0.765, *P*= 0.004) and *Trichoderma* spp. population (r= 0.697, *P*= 0.012) but it was significantly and negatively (r= -0.700, *P*= 0.01) linked to the total culturable fungal population in the analyzed soil samples (Fig. 3).

Pearson correlation analysis, also, revealed a significant and negative correlation between the average fruit yield per plant and the fungal population (r = -0.701; *P* = 0.011) colonizing the rhizosphere of pumpkins accessions (Fig. 3).

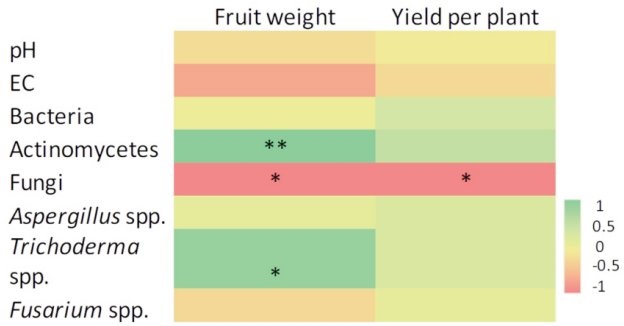

Fig. 3 - Heat map of Pearson's correlation (r) between the average fruit weight and the average yield per plant of pumpkins accessions and soil characteristics. Asterisks indicate statistically significant correlation values, negative or positive at * P≤0.05 and ** P≤0.01.

Multicriteria analysis via PCA

Based on the PCA analysis performed, the first two main components (PC) comprised about 68.31% of the variability existing in the analyzed genotypes. PC-1 explained 47.05% of the total variability. The most important traits related to this axis were: the fruit fresh weight, the yield per plant, and actinomycetes and *Trichoderma* spp. population. The most important traits of PC-2, which explained 21.25% of the total variation, were EC values and *Aspergillus* spp. community (Fig. 4A).

The distribution of pumpkins accessions among the two axes showed the variability and allowed distinguishing 3 main groups (Fig. 4B). The 1st group included 4 accessions (C5, C23, C14.2 and C6.2 belonging to *C. maxima*) characterized by the highest average fruit weight, the highest yield per plant, the

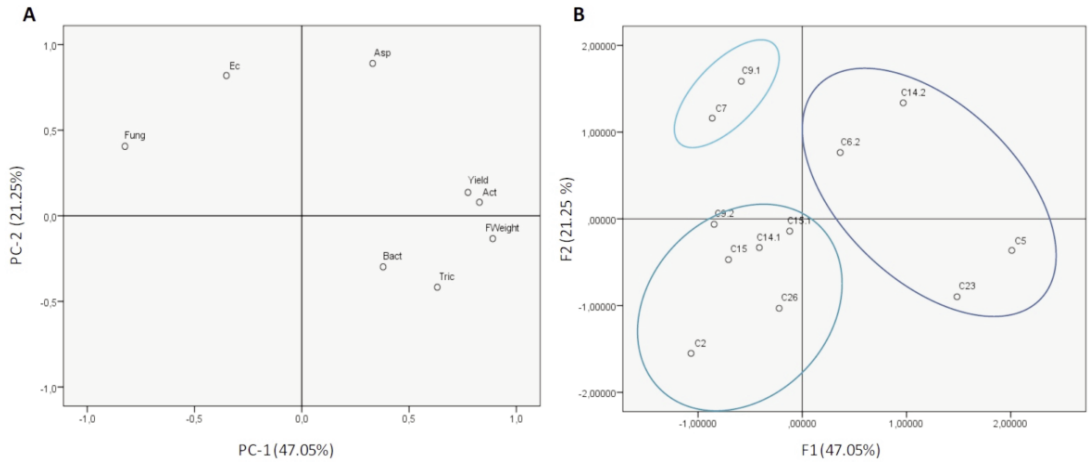

Fig. 4 - PCA biplot the variability existing in the analyzed traits (A) and the distribution of pumpkins accessions (B). Ec: Electrical conductivity. Fung: Fungi. Asp: *Aspergillus* spp. Yield: Yield per plant. Act: Actinomycetes. Fweight: Fruit weight. Bact: Bacteria. Tric: *Trichoderma* spp. C7 and C15: *Cucurbita pepo*. C2, C5, C6.2, C9.1, C9.2, C14.2, C15.1 and C23: *C. maxima*. C14.1 and C26: *C. moschata.*

highest actinomycetes and bacterial communities, the highest *Trichoderma* spp. and *Aspergillus* spp. populations, and the lowest fungal community in their rhizosphere. The 2nd group was comprised of C7 (*C. pepo*) and C9.1 (*C. maxima*) accessions characterized by the lowest fruit weight and the lowest *Trichoderma* spp. populations, and the 3rd group was composed of the remaining 6 accessions exhibiting intermediate yield per plant and *Trichoderma* spp. populations.

4. Discussion and Conclusions

Plant-associated microbiome plays a fundamental role in plant growth and health (Wei and Joussset, 2017). Breeding programs focusing genotype-associated beneficial microbiome help achieve ecologically desired plant phenotype traits (Adam *et al*., 2018; Wille *et al*., 2018). The current study aimed to select the most productive pumpkins accessions based on the variability of their soil microbial community structure and to investigate the presence of eventual links between *Cucurbita* spp. production and yield parameters and their rhizosphere soil associated microorganisms. Our results clearly demonstrated that *Cucurbita* species and accessions shaped their own soil microbial community structure. Some microbes have a particular affinity for certain pumpkins accessions in determining rhizosphere communities. The variation of composition of microbial distribution in the rhizosphere of *Cucurbita* spp. accessions may be explained by the differences in their root morphology and the composition and content of their root exudates which play a fundamental role in the recruitment of plant holobiont. Plant-associated microbiome and their interactions are highly diverse and multiple factors shape the microbial community assembly and functioning. In fact, the microbial community's structure varies significantly depending on plant species and/or genotypes growing in the same soil environment (Kang and Mills, 2004; Yao and Wu, 2010; Berendsen *et al*., 2012; Aydi Ben Abdallah *et al*., 2023) and even on plant growth stage (Chaparro *et al*., 2014; Compant *et al*., 2019). The variation in the soil-associated microbiome communities has been assigned to the differences in the root morphology, the type of rhizodeposits, the amount and the composition of root exudates and mainly carbon sources which are limiting factors for microbial activity and proliferation (Marschner *et al*., 2007; Broeckling *et al*., 2008; Compant *et al*., 2019). Moreover, edaphic factors such as soil pH, electrical conductivity (EC), soil texture, soil parental material, and soil salinity are important determinants of community structure and diversity of soil microbiome (Lozupone and Knight, 2007; Lauber *et al*., 2008; Xu *et al*., 2014; Sun *et al*., 2015; Min *et al*., 2016).

The soil-associated microbiomes have an effect on plant growth and yield production. Positive and significant correlations were determined between fruit fresh weight and the culturable bacterial and *Trichoderma* spp. populations in the rhizopshere of pumpkins accessions tested in the current investiga-

tion. Plant-associated microbes with their plant growth-promoting traits play a crucial role in enhancing plant biomass and crop yield (Kumar *et al*., 2022). Halifu *et al*. (2019) demonstrated that inoculation with two *Trichoderma* species on *Pinus Sylvestris* var. *mongolica* seedlings had a positive correlation with growth parameters, soil nutrient content, and soil enzymatic activity in their rhizosphere. *Trichoderma* spp. are able to increase the growth and the extension of the root system and to stimulate the secretion of extracellular enzymes such as sucrase, urease, phosphatase, and organic acids in the rhizosphere. These compounds lead to the improvement of the nutrient cycle and the soil enzymatic activity and consequently the soil nutrient status and availability (Pelagio-Flores *et al*., 2017). Furthermore, *Trichoderma* spp. can secrete the indole 3 acetic acid (IAA) and to promote the growth of many crops as previously demonstrated for cucumber, bottle gourd, and bitter gourd (Kotasthane *et al*., 2015). Furthermore, the volatile and non-volatile secondary metabolites released by *Trichoderma* spp. such as 6-n-pentyl-6H-pyran-2-one (6PP), gliotoxin, viridin, harzianopyridone, harziandione, and peptaibols have a significant growth-promoting effect on plants (José *et al*., 2008). Mohanty *et al*. (2021) also demonstrated that the beneficial bacterial communities may improve crop productivity as part of sustainable agriculture. In fact, *Acidothiobacillus ferooxidans* and *Bacillus cereus* are associated to increased growth and yield and improved soil composition in pumpkin (Ansari *et al*., 2017).

Plant growth improvement may be achieved either directly via the enhancement of nutrient availability and phytohormone modulation and/or indirectly through the biocontrol activity i.e. suppression of associated pathogens and/or the alleviation of biotic and abiotic stresses leading to the improvement of both plant health and crop productivity (Khan *et al*., 2020; Basu *et al*., 2021; Zhang *et al*., 2021; Kumar *et al*., 2022). In the current study, negative and significant correlations were noted between the average fruit weight and yield per plant parameters and the total culturable fungal populations. The fungal population estimated in the rhizosphere of pumpkins accessions may be mainly composed of soilborne pathogens naturally associated to pumpkins plants which may be involved in the recorded decreases in fruit weight and yield. Based on ACP analyses, the 1st group was comprised of 4 accessions of *C. maxima* (namely C5, C23, C14.2 and C6.2) which are characterized with the highest production parameters (average fruit weight and yield per plant) and the highest populations of actinomycetes, bacteria, *Trichoderma* spp. and *Aspergillus* spp., and also the lowest fungal community in their rhizosphere. Hence, these four microbial groups (bacteria, *Aspergillus* spp., *Trichoderma* spp. and actinomycetes) predominant in the rhizosphere of these 4 most productive pumpkins accessions may be involved, either individually or in consortium, indirectly in the promotion of pumpkins yield via their eventual antagonistic potential against their associated fungal pathogens. As demonstrated in Yang *et al*. (2017) study, some potential plant-beneficial microbial agents could act as network key, thus reducing the chance of a given a soil-borne pathogen to invade the target plant species. Also, Chaurasia *et al*. (2018) demonstrated the successful role of actinomycetes on plant protection and growth promotion of Solanaceae, Cucurbitaceae, Brassicaceae, Amaranthaceae, Umbelliferous, Asteraceae, Fabaceae, Asparagaceae, and Amaryllidaceae vegetable crops. Also, as demonstrated in Hung and Rutgers (2016) study, *Aspergillus* spp. are multifaceted fungi that the plant benefits with different manner such as plant growth promotion and protection. Pascual *et al*. (2017) also emphasized the role of *T. harzianum* in reducing the natural infection of melon plants by *F. oxysporum* f. sp. *melonis* and in improving their yields. In Aydi Ben Abdallah *et al*. (2019) study, *Bacillus subtilis* SV41 and *B. amyloliquefaciens* subsp. *amyloliquefaciens* SV65 have successfully decreased the soil infection potential by *Fusarium* species, suppressed Fusarium wilt severity and enhanced tomato growth and production.

In our study, the 4 selected pumpkins accessions are quite able to exploit their associated beneficial indigenous microbial communities which could be considered in the future pumpkin breeding programs.

In conclusion, this study clearly demonstrated the significant role of tested accessions in affecting the distribution of microbial community in their rhizosphere leading to differences in yield parameters between pumpkins accessions. The variation in the microbial community structure with the accessions tested might be due to the changes in the composition of their root exudates which need to be more elucidated in our future investigations. Four accessions of *C. maxima* (namely C5, C23, C14.2 and C6.2) have a great potential as they are characterized by the highest average fruit weight and yield per plant,

the highest populations of actinomycetes, bacteria, *Trichoderma* spp. and *Aspergillus* spp., and the lowest fungal population in their rhizosphere. Thus, the exploitation and the re-integration of the recovered beneficial bacterial and *Trichoderma* spp. populations associated with these four selected accessions of *C. maxima* will be considered in the future pumpkin breeding programs to reduce the threat imposed by their soil-borne pathogens and consequently led to more enhancements in pumpkin fruit yield into the less productive accessions.

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Shallot cultivation in tropical climate ecosystems using floating and non-floating systems with different doses of cow manure

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Abstract: Deep swamp is swampland with the longest flooding period, making it challenging for crop cultivation. However, by adopting a floating system, this prolonged duration of flooding can be used for shallot growing. Thus, this study aimed to ascertain the growth and yield of shallots cultivated in polybags using conventional non-floating and floating systems with the application of different doses of cow manure. The research was located in the experimental field and reservoir of the Faculty of Agriculture, Sriwijaya University (3°13'30.3'' S; 104°38'55.1" E). Non-floating and floating farming systems were utilized with the application of 0, 10, 15 and 20 ton/ha of cow manure. The findings demonstrated that shallots cultivated in the floating system had lower numbers and length of leaf but could produce more bulbs in comparison to the conventional method. The application of 15 ton/ha manure in the floating system resulted in higher weights of fresh and air-dried bulbs per plant, weighing 74.40 g and 64.82 g, respectively, compared to those in the non-floating system (46.77 g and 37.84 g, respectively). In conclusion, the Bima Brebes shallot variety potentially can be cultivated in a floating system with the application of 15 tons of cow manure per hectare.

1. Introduction

As one of the strategic commodities widely consumed in Indonesia, shallot (*Allium ascalonicum* L.) is a vegetable crop that significantly contributes to the country's horticultural production and inflation rate. According to the findings of the Socio-Economic Survey in September 2021, Indonesians consume an average of 2.49 kg of shallots per person each month. Shallots are required for the food sector, where they are processed into ready-to-use seasonings for sprinkling on food dishes, as well as for usage in households as a seasoning for cooking (Ministry of

Agriculture, 2019; Irjayanti, 2022).

The amount of shallots needed for household consumption and the food industry continues to increase. The government's involvement in meeting these needs is through a program to organize and grow shallot production centers outside Java Island so that production centers are not just concentrated on Java. This program aims to realize shallot self-sufficiency in every province in Indonesia (Ministry of Agriculture, 2019; Indriyana *et al*., 2020). South Sumatra is one of the provinces targeted by this program; this is because shallot production in South Sumatra is still low at only 0.057% of national production or 1125 tons in 2021 (Central Bureau of Statistics for South Sumatra Province, 2022; Directorate of Statistical Dissemination, 2022). Even though South Sumatra is a lowland region that is suitable for growing shallots, there are still several challenges that may affect the shallot growth. One of these is the land's condition as swampland, particularly *lebak* swampland.

Lebak swampland, with its alluvial soil type, has considerable potential to increase the production of food and horticultural crops. However, the use of *lebak* for crop cultivation is faced with high-water fluctuations that cause flooding in the rainy season and drought in the dry season. The typology of *lebak* swampland based on the height and duration of standing water is divided into shallow, middle and deep swamp. Once or twice a year, rice can be grown in the shallow and middle swamps. During the dry season, horticultural crops, particularly vegetable crops, can also be grown, although there is a risk from drought (Djafar, 2013; Suprapto, 2016; Suryana, 2016; Widuri *et al.*, 2016; Pujiharti, 2017; Simatupang and Rina, 2019). Deep swamp is an inland swamp area with stagnant water for more than six months and even during the dry season it remains stagnant. As a result, cultivating plants becomes quite challenging. The deep swamp is mostly left unutilized during the high flooding period. Utilizing a floating cultivation technique is one option for making use of this area (Siaga *et al.*, 2018; Jaya *et al.*, 2019; Lakitan, 2021; Susilawati *et al.*, 2023). According to Hasbi *et al.* (2017), a projected floating farming system for the cultivation of vegetables was created based on the statements of farmers who are interested in using the newly introduced floating farming. Shallots are one of the many crops that may be grown in the floating system.

One of the factors that affect the growth of shallots is the planting medium used. The texture and structure of the soil have significant impact on the production and quality of shallots. Applying organic fertilizer will create the fertile, loose soil that shallots need for the development of their bulbs. One of the components that can increase the physical, chemical and biological qualities of the soil to boost the productivity of shallot plants while reducing the amount of phosphorus (P) fertilizers added to P-deficient soils is organic fertilizer (Noviyanty and Salingkat, 2018; Susikawati *et al.*, 2018; Nguyen *et al.*, 2021). One organic fertilizer that can promote plant development is cow manure (Sudarsono *et al.*, 2014; Atman *et al.*, 2018; Musdalifah *et al.*, 2021). Thus, this research was conducted with the aim of evaluating the growth response and production of shallot plants cultivated in polybags using non-floating and floating systems with the application of various doses of cow manure fertilizer.

2. Materials and Methods

Research gate

The research was located in the experimental field and reservoir of the Faculty of Agriculture, Sriwijaya University, Indralaya Ogan Ilir (3°13'30.3''S; 104°38'55.1"E). Figure 1 shows the arrangement during the dry season in tropical climate ecosystems of South Sumatra, Indonesia, from May to August 2022. Typical agroclimatic conditions at the outdoor research facilities are shown in figure 2.

Procedures

The shallot bulbs used were of the Bima Brebes variety originated from the shallot seed farmers in Brebes, Central Java. The experiment was arranged using a factorial randomized block design with two factors and three replicates. The treatments consist-

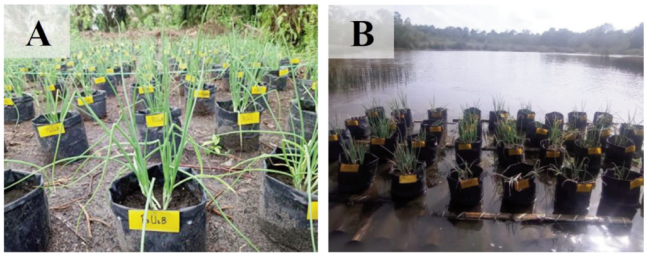

Fig. 1 - Non-floating (A) and floating (B) farming practices of shallot cultivation.

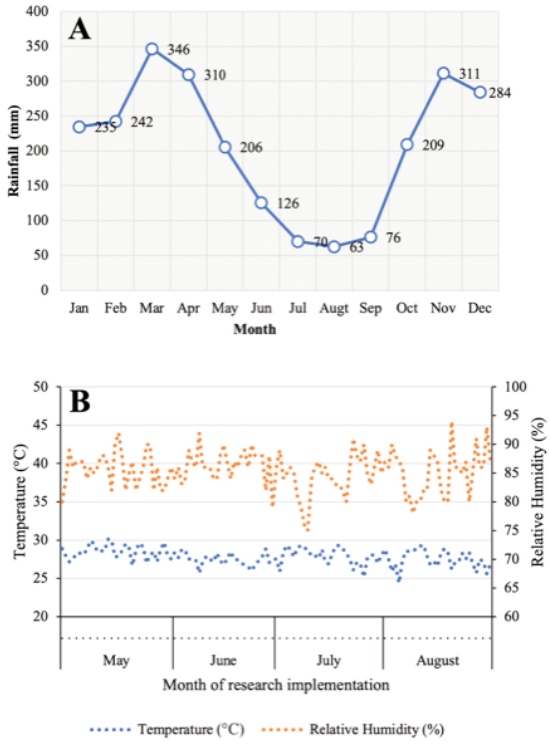

Fig. 2 - Typical agroclimatic conditions at the outdoor research facilities: (A) rainfall; (B) temperature and relative humidity. Source: https://www.bmkg.go.id.

ed of different farming practices (conventional non-floating and floating systems) and dosages of cow manure (0, 10, 15 and 20 ton/ha).

For all farming practice treatments, the planting media were prepared using the same method: after being completely mixed, they was placed into 35 cm x 30 cm polybags. The planting media were a mixture of alluvial topsoil gathered from *lebak* swamp combined with cow manure according to the treatments. In the conventional farming technique, the area was prepared by clearing the weeds to make a space for placing the polybags filled with the media. The planting space was 20 cm x 20 cm, following the recommendation for shallot cultivation. In the floating cultivation, the polybags were put on a 2 m x 1 m bamboo raft. Each replicate was put on one bamboo raft. The planting media were sprayed with Bio Soil Grow Booster at a concentration of 4 ml/L of water one week before planting. Inorganic fertilizers were also used, with dosages of 69 kg/ha of P_2O_5, 46 kg/ha of N and 60 kg/ha of K_2O. Phosphorus fertilizer was applied 7 days before planting, whereas nitrogen and potassium were applied twice, 7 and 25 days after planting, each time in half the prescribed amount. Before being planted, the top one-third of the bulb was cut off and the bulb was placed into a planting hole at depth 2-3 cm. Shallot plants cultivated in the non-floating system were watered regularly to ensure sufficient water availability. In the floating system, the plants were not watered because water was continuously supplied through the soil pores by capillary force from the swamp water below.

Data analysis

The variables were growth characteristics such leaf length, leaf number and leaf color, shallot production variables such as bulb number, bulb diameter, fresh weight, dry weight and shrinkage percentage, as well as estimated production per hectare. Leaf color was measured using a chlorophyll meter (SPAD-502, Minolta) to estimate leaf greenness level correlated to the chlorophyll content (SPAD value). Growth parameters were observed every week, whereas the production data were gathered after the harvest. The collected data were analyzed using R Studio statistical analysis software. The calculated F-value generated from the analysis of variance (ANOVA) was compared to values at $p \leq 0.05$ and $p \leq 0.01$ for justifying the significant effects of the treatments. Furthermore, if the treatment effect was significant for any measured trait, the least significant difference (LSD) test was conducted to determine significant differences among treatment levels for each specified trait.

3. Results and Discussion

Leaf length (cm) and leaf number of shallots

Shallot plants grown in tropical climate ecosystems using two farming practices - conventional non-floating (Fig. 1A) and floating (Fig. 1B) systems with various doses of cow manure - showed differences in growth and yield. Rainfall continued to decline from May to August 2022, the period when the study was conducted. The rainfall reduced from 206 mm in May to 63 mm in August (Fig. 2A). High rainfall levels at the start of the study provided a favorable environment for shallot growth in the non-floating cultivation system (polybags stacked on dry soil). On the other hand, shallots grown in wetlands using the floating system (polybags placed on rafts) did not favor heavy rainfall. From May to August 2022, the temperature and humidity remained relatively stable at 27.47-27.92°C and 84.51-86.36%, respectively (Fig. 2B).

ANOVA revealed that the variations in farming practices had a significant impact. The results of the LSD test on leaf length demonstrated that the two farming practices differed significantly. In conventional cultivation, the maximum leaf length was 40.43±1.25 cm at 5 weeks after planting (WAP), whereas in the floating system it was only 30.47±0.47 cm at 4 WAP. In the floating agriculture system, the position of the plants was adjusted so that they would always be waterlogged to a height of about 3 cm from the base. In that case, water was continuously supplied via the soil pores by capillary force, causing slower oxygen diffusion. Additionally, when it rains heavily, the media become more water-saturated, which lowers the amount of accessible oxygen. Oxygen is needed by the roots for respiration and for maintaining healthy cell function (Neira *et al*., 2015; Ernest, 2018; Jaya *et al*., 2021; Kartika *et al*., 2021). Damage to the root will eventually affect the upper plant growth, as seen from the agronomical features. Research by Susilawati *et al.* (2012) on red pepper plants showed that all cultivars experienced varying degrees of root damage as a result of flooding stress. The amount of oxygen present in the planting media is significantly influenced by the height of the water table. Research on bean plants has shown that the roots, particularly the process of root respiration, were significantly impacted by water levels that were 10 cm below the surface of the planting media. Although organic fertilizer applied to shallots in a floating system did not affect growth, a proper water level and the application of organic matter to shallot plants considerably stimulated growth (Susilawati and Lakitan, 2019; Susilawati *et al*., 2019, 2022). With regard to leaf length, the application of cow manure showed insignificant results in the first week but significant results at 2–8 WAP. The longest leaves (40.19 ± 2.96 cm) were obtained at a P_3 treatment dose of 20 ton/ha, which is not significantly different from the 40.06 ± 1.69 cm obtained at a P_2 dose of 15 ton/ha. The combination of farming practices and cow manure treatments had a significant effect on leaf length only at 6 and 8 WAP in the conventional P_3 treatment, with lengths of 44.74 ± 1.10 cm and 44.92 ± 1.36 cm, respectively.

For leaf number, the difference in farming practices only had a significant effect in the first three weeks. The average number of leaves was mostly higher in the conventional system, except in the fourth week when the leaf number in the floating system was higher at 24.33 ± 1.92 compared to 23.39 ± 1.33 in the conventional system. The research on eggplant showed that increasing the water content of the substrate from 1 to 3 cm would increase the growth of vegetative organs (Jaya *et al.*, 2019). The difference in cow manure dose affected the number of leaves, with the highest leaf number of 31.78 ± 2.65 obtained in P_2 treatment at 7 WAP (Table 1).

Similarly, research by Feriatin *et al.* (2021) also showed that the use of cow manure would affect leaf number of the Lokananta shallot variety. The P_3 treatment for conventional cultivation and the P_2 treatment for floating cultivation produced the highest average leaf length and leaf number when cow manure was applied. In the conventional P_3 treatment, the maximum leaf length was 37.45 cm with an average of 21.33 leaves, while in the floating P_2 treatment the highest leaf length was 29.80 cm with 23.14 leaves. Cow manure is an organic fertilizer that can alter the structure and texture of the soil, making the media crumblier, which explains the difference in the dosage of cow manure between the two cultivation systems (Fig. 3). Meanwhile, as a result of the relatively high moisture of the planting media in the floating culture, cow manure already affects the texture and structure of the media at lower doses (Elisabeth *et al.*, 2013; Gudugi, 2013; Ekwealor *et al.*, 2020; Wisdom *et al.*, 2021).

SPAD value

In this study, the parameter of leaf greenness - which serves as an indicator for chlorophyll content - was quantified using the SPAD tool without damaging the leaves measured from the second to the eighth week. At 4 and 6 WAP, the culture technique treatment significantly affected the SPAD value but had no significant effect at 2 and 8 WAP. Shallots cultivated in the floating system were recorded to have higher values at 2, 4 and 6 WAP (77.69±2.69, 64.10± 4.41 and 48.18 ± 1.25, respectively) compared to those in the conventional system (74.68±3.08, 48.18±1.25 and 48.79±1.43). However, at 8 WAP, the SPAD value in the conventional system was higher than in the floating system (44.78±1.33 vs. 43.22±4.48). The SPAD value of the two cultivation techniques was at its highest at 2 WAP and then continued to decrease until 8 WAP. In comparison to other models, the polynomial model's regression analysis of the SPAD value in the shallot cultivation resulted in the largest determination coefficient (R^2), which is close to 1: 0.9123 for the conventional system and 0.9618 for the floating system. The magni-

Table 1 - Leaf length (cm) and leaf number of shallot with the application of cow manure (ton/ha) in different farming practices

Treatment	Weeks after planting (WAP)							
	1	2	3	4	5	6	7	8
Farming practice				*Leaf length (cm)*				
Conventional	11.67 ± 0.66 a	26.60 ± 0.79 a	36.43 ± 1.03 a	40.32 ± 1.45 a	40.43 ± 1.69 a	38.39 ± 1.71 a	38.39 ± 1.66 a	36.09 ± 1.95 a
Floating	8.28 ± 0.32 b	20.04 ± 0.56 b	27.58 ± 1.09 b	30.47 ± 1.43 b	29.45 ± 1.90 b	26.66 ± 2.08 b	25.66 ± 1.82 b	24.88 ± 1.76 b
Significance	**	**	**	**	**	**	**	**
LSD0.05	1.516	1.375	2.209	1.961	1.992	2.208	2.536	4.549
				Leaf number				
Conventional	7.83 ± 0.39 a	12.38 ± 0.43 a	17.78 ± 0.69 a	22.13 ± 0.93	23.39 ± 1.33	25.69 ± 1.50	26.00 ± 1.43	23.64 ± 1.21
Floating	5.88 ± 0.21 b	10.38 ± 0.47 b	15.66 ± 0.61 b	20.94 ± 1.25	24.33 ± 1.92	25.25 ± 2.15	23.61 ± 2.06	20.03 ± 1.91
Significance	**	**	*	ns	ns	ns	ns	ns
LSD0.05	0.777	1.303	1.548	2.479	2.929	3.831	4.679	4.567
Cow manure (ton/ha)				*Leaf length (cm)*				
P_0 (0)	9.31 ± 0.90	21.37 ± 1.74 b	27.53 ± 2.37 b	29.07 ± 2.54 b	26.80 ± 2.99 c	24.83 ± 3.09 c	25.61 ± 3.07 c	25.24 ± 2.96 b
P_1 (10)	10.19 ± 1.23	22.09 ± 1.24 b	30.71 ± 1.86 a	33.59 ± 2.21 b	32.72 ± 2.64 b	29.26 ± 3.12 b	29.86 ± 3.00 b	30.29 ± 2.58 b
P_2 (15)	9.54 ± 0.87	25.30 ± 1.58 a	35.52 ± 1.81 a	39.69 ± 1.74 a	40.06 ± 1.69 a	38.93 ± 1.33 a	37.84 ± 1.34 a	37.16 ± 1.80 a
P_3 (20)	10.86 ± 1.12	24.52 ± 1.89 a	34.26 ± 2.31 a	39.23 ± 2.82 a	40.19 ± 2.96 a	37.10 ± 3.46 a	34.76 ± 4.63 a	29.27 ± 4.69 b
Significance	ns	**	**	**	**	**	**	*
LSD0.05	2.144	1.945	2.189	2.773	2.817	3.122	3.587	6.434
				Leaf number				
P_0 (0)	6.00 ± 0.40 b	10.33 ± 0.31	14.33 ± 0.41 b	17.55 ± 0.62 b	17.72 ± 0.88 c	19.50 ± 1.64 c	20.50 ± 2.11 b	19.39 ± 2.28
P_1 (10)	6.94 ± 0.59 ab	11.33 ± 1.06	16.78 ± 1.11 a	20.39 ± 1.12 b	22.50 ± 1.32 b	25.99 ± 1.47 b	26.28 ± 2.09 ab	23.11 ± 2.04
P_2 (15)	6.72 ± 0.58 ab	11.39 ± 0.76	17.22 ± 0.99 a	24.00 ± 1.59 a	29.05 ± 2.38 a	31.78 ± 2.65 a	29.67 ± 2.39 b	25.94 ± 1.88
P_3 (20)	7.78 ± 0.68 a	12.50 ± 0.55	18.55 ± 0.59 a	24.22 ± 0.80 a	26.16 ± 1.32 ab	24.61 ± 1.78 bc	22.78 ± 2.14 b	18.89 ± 2.36
Significance	*	ns	**	**	**	**	*	ns

Data represent the mean and standard error. Values followed by different letters within each column indicate a significant difference at LSD 0.05.

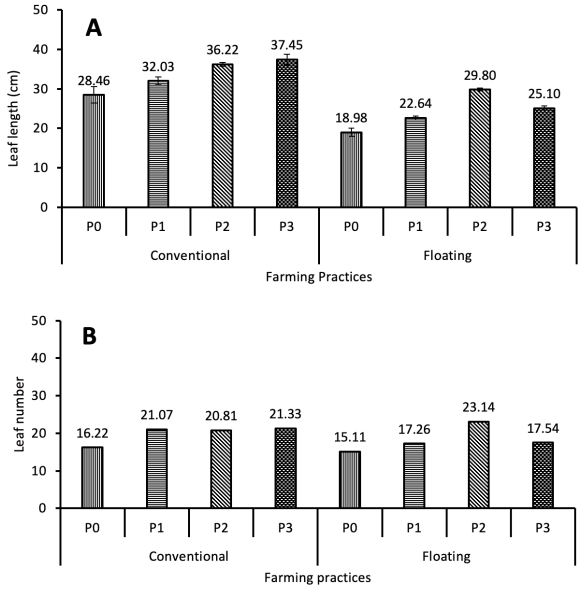

Fig. 3 - Effect of cow manure dosage on leaf length (A) and leaf number (B) in the different farming practices.

tude of the R^2 value indicates that the SPAD value is affected by the cultivation technique in a quadratic manner, increasing until it reaches the peak before starting to decline (Table 2). A study on corn resulted in a similar result, where the SPAD values would decrease after reaching their peak, mostly affected by the environment (Ghozali, 2016; Kandel, 2020; Szulc *et al.*, 2021).

The higher SPAD values in the floating system, especially at 4 and 6 WAP, indicated that the photosynthesis process is going well due to sufficient water availability. In contrast, if there is a deficit of water (moisture stress), photosynthetic activity will be reduced due to chlorophyll damage (Pallavolu *et al.*, 2023). The research results of Ai Nio *et al.* (2019) showed that the water deficit induced by PEG 8000 with media water potential (WP) -0.25 and -0.5 MPa reduced the total leaf chlorophyll content, leaf chlorophyll *a* and leaf chlorophyll *b*.

This study also found that the increase in cow manure dosage increases the SPAD value, with the

Table 2 - Regression analysis correlations of the SPAD value with several mathematical models of farming practices using different doses of cow manure

Farming practice	Linear model y = ax + b	Logarithmic model y = a ln x + b	Polynomial model y = ax² + bx +c	Power model y = ax^b
Conventional	y = –8.9073*x* + 76.38	*y* = –21.1ln(*x*) + 70.877	*y* = 5.6238*x*²–37.026*x* +	*y* = 70.35*x*^0.357
	R^2 = 0.6917	R^2 = 0.8417	R^2= 0.9123	R^2 = 0.8842
	r = 0.8316**	*r* = 0.9174**	*r* = 0.9551**	*r* = 0.9432**
Floating	y =-10.769*x* + 88.122	*y* = –22.56ln(*x*) + 79.122	*y* = –0.7451*x*²–7.0429*x* +	*y* = 80.712*x*^0.375
	R^2 = 0.9581	R^2 = 0.9116	R^2 = 0.9618	R^2 = 0.8804
	r = 0.9788**	*r* = 0.9547**	*r* = 0.9807**	*r* = 0.9383**

** Significant difference at p < 0.05.

highest SPAD value of 82.54 obtained in the P_3 treatment and the lowest (31.21) in the P_0 treatment. The SPAD value has been widely used to estimate the chlorophyll content of other crops, such as tomatoes (Jiang *et al.*, 2017). Furthermore, the application of cow manure could also increase leaf chlorophyll, as indicated by the SPAD value in wheat and rice plants (Shah *et al.*, 2017; Atman *et al.*, 2018). The combination of cultivation techniques and cow manure treatments resulted in no significant effect at 2 and 8 WAP, a significant effect at 4 WAP and a highly significant effect at 6 WAP. The highest SPAD values were obtained in the floating cultivation with a cow manure dose of 20 ton/ha: 77.01 ± 5.35 at 4 WAP and 75.11 ± 3.96 at 6 WAP (Table 3).

Bulb number, bulb diameter (mm) and weight of fresh and air-dried bulbs (g)

The yield components include the number of bulbs, bulb diameter and weight for both fresh and

Table 3 - The SPAD value of shallot in different farming practices with the application of cow manure

Treatment	SPAD value			
	2 WAP	4 WAP	6 WAP	8 WAP
Farming practice				
Conventional	74.68 ± 3.08	48.18 ± 1.25 b	48.79 ± 1.43 b	44.78 ± 1.33
Floating	77.69 ± 2.88	64.10 ± 4.41 a	59.78 ± 4.55 a	43.22 ± 4.48
Significance	NS	**	**	ns
LSD value	7.996	6.040	4.381	4.615
Cow manure (ton/ha)				
P_0 (0)	68.16 ± 2.38	45.31 ± 2.88 c	40.90 ± 2.71 c	31.21 ± 4.08 c
P_1 (10)	75.74 ± 5.19	58.48 ± 5.77 ab	56.21 ± 4.35 b	44.61 ± 2.93 b
P_2 (15)	78.27 ± 4.09	55.78 ± 4.94 b	55.98 ± 3.73 b	46.60 ± 2.09 b
P_3 (20)	82.54 ± 2.98	65.00 ± 6.08 a	64.04 ± 5.28 a	53.60 ± 3.80 a
Significance	NS	**	**	**
LSD value	11.307	8.542	6.197	6.527
Farming practice x Cow manure (ton/ha)				
Conventional x P_0	68.47 ± 5.10	46.81 ± 1.43 b	43.29 ± 3.72 ef	39.69 ± 3.16 c
Conventional x P_1	74.13 ± 10.00	46.93 ± 1.68 b	48.02 ± 1.74 de	43.42 ± 10.00 bc
Conventional x P_2	74.01 ± 4.26	46.00 ± 1.36 b	50.87 ± 0.68 bc	46.57 ± 4.26 bc
Conventional x P_3	82.13 ± 4.21	52.99 ± 3.47 b	52.98 ± 1.17 cd	49.46 ± 4.21 ab
Floating x P_0	67.86 ± 1.46	43.81 ± 6.11 b	38.52 ± 4.15 f	22.73 ± 1.46 d
Floating x P_1	77.37 ± 5.68	70.03 ± 5.53 a	64.41 ± 4.95 b	45.81 ± 5.68 bc
Floating x P_2	82.54 ± 6.90	65.56 ± 4.94 a	61.10 ± 6.57 bc	46.63 ± 6.90 bc
Floating x P_3	82.97 ± 5.15	77.01 ± 5.35 a	75.11 ± 3.96 a	57.73 ± 5.15 a
Significance	NS	*	**	NS
LSD value	15.991	12.081	8.763	9.231

WAP= Week after planting.
Data represent the mean and standard error. Values followed by different letters within each column indicate a significant difference at LSD 0.05.

air-dried bulbs. The farming practice had no significant impact on the quantity of bulbs but a very significant impact on the weight of fresh and air-dried bulb and the bulb diameter. The conventional method produced the greatest number of bulbs, whereas floating cultivation produced the best results in terms of bulb diameter and weight of fresh and air-dried bulbs. The growing media conditions strongly affected how the bulbs were initially formed. Since water was constantly accessible from beneath the growing media through capillaries, floating cultivation used growing media that were somewhat moist. There was also intense rainfall during early growth of the shallots, causing the planting media to be very wet. There were about 7.78 ± 0.35 bulbs formed in conventional cultivation and 7.75 ± 0.33 in the floating cultivation. However, the number of bulbs was not linearly correlated with the greater dose of cow manure applied, as the largest number of bulbs was obtained at a dose of 15 ton/ha (Table 4). The largest numbers of bulbs (8.33 in conventional cultivation and 8.22 in floating cultivation) were obtained from the same manure treatment, which was P_2. The lowest numbers (6.78 in conventional cultivation and 6.67 in floating cultivation) were also obtained from the same manure treatment, P_0. Plant growth can thus be supported by appropriate cultivation methods (Khorasgani and Pessarakli, 2019; Cahyaningrum *et al.*, 2023).

The average diameter of bulbs grown using floating cultivation was 23.51 ± 1.29 mm, which is much larger than the 20.62 ± 1.62 mm average diameter of bulbs grown using conventional cultivation (Fig. 4). The largest diameter of bulbs produced as a result of cow manure application was at a dose of 15 ton/ha, and the lowest was at 0 ton/ha (Table 4). Based on the combination of treatments, P_2 treatment (15 ton/ha) in floating cultivation produced the largest bulb diameter of 30.78 mm, and P_3 treatment (20 ton/ha) in conventional cultivation produced the largest bulb diameter of 25.36 mm. The two cultivation methods produced the smallest bulb diameters in the same treatment, P_0 (0 ton/ha), with the conventional system producing a diameter of 15.22 mm

Table 4 - Shallot yield components for different farming practices with the application of cow manure

Treatment	Number of bulbs	Diameter of bulb (cm)	Fresh weight of bulb (g)	Air-dried weight of bulb (g)
Farming practice				
Conventional	7.78 ± 0.35	20.62 ± 1.76 b	35.36 ± 6.14 b	26.79 ± 5.64 b
Floating	7.75 ± 0.33	23.51 ± 1.29 a	42.33 ± 3.52 a	35.69 ± 2.94 a
Significance	NS	**	*	**
LSD value	1.016	1.741	6.419	4.703
Cow manure				
P_0 (0)	7.44 ± 0,45	15.44 ± 0.77 c	20.57 ± 2.85 c	14.71 ± 2.12 d
P_1 (10)	7.44 ± 0.50	19.88 ± 0.84 b	34.46 ± 2.36 b	25.64 ± 1.49 c
P_2 (15)	8.28 ± 0.53	27.10 ± 1.74 a	58.24 ± 7.76 a	48.22 ± 7.75 a
P_3 (20)	7.89 ± 0.44	25.84 ± 0.81 a	42.11 ± 2.94 b	36.39 ± 1.84 b
Significance	NS	**	**	**
LSD value	1.437	2.461	9.078	6.651
Farming practice x Cow manure				
Conventional x P_0	6.78 ± 0.72	15.22 ± 1.40 e	19.58 ± 4.90 e	13.15 ± 3.18 f
Conventional x P_1	8.22 ± 0.58	18.49 ± 0.98 de	33.01 ± 3.72 cd	24.57 ± 1.99 de
Conventional x P_2	8.33 ± 0.76	23.43 ± 0.81 bc	42.08 ± 1.87 bc	31.61 ± 0.73 bcd
Conventional x P_3	7.78 ± 0.44	25.36 ± 1.03 b	46.77 ± 4.21 b	37.84 ± 0.89 b
Floating x P_0	8.11 ± 0.22	15.66 ± 0.99 e	21.57 ± 3.95 de	16.28 ± 3.15 ef
Floating x P_1	6.67 ± 0.57	21.28 ± 0.80 cd	35.92 ± 3.45 bc	26.71 ± 2.44 cd
Floating x P_2	8.22 ± 0.90	30.77 ± 1.02 a	74.40 ± 6.05 a	64.82 ± 4.92 a
Floating x P_3	8.00 ± 0.88	26.32 ± 1.40 b	37.45 ± 1.96 b	34.96 ± 2.56 b
Significance	NS	*	**	**
LSD value	2.032	3.481	12.838	9.406

WAP= Week after planting.
Data represent the mean and standard error. Values followed by different letters within each column indicate a significant difference at LSD 0.05.

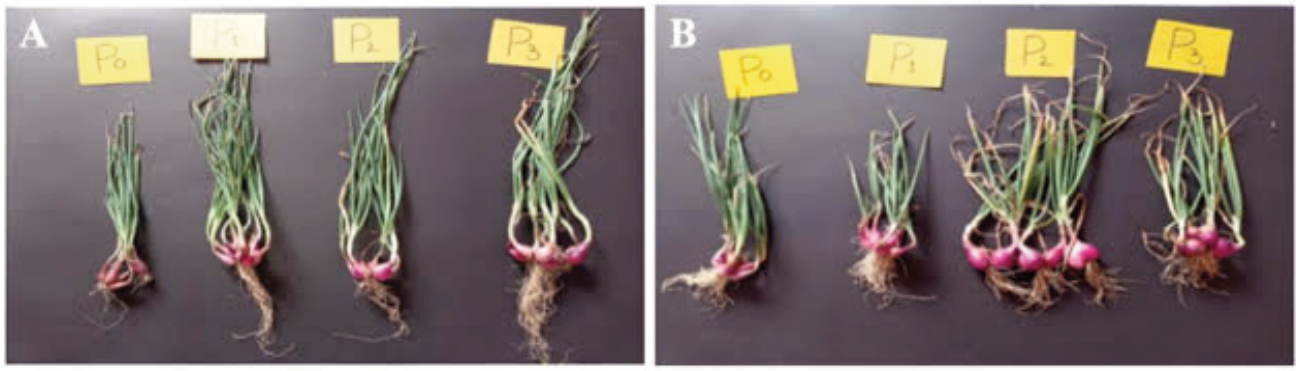

Fig. 4 - Shallot bulbs produced by the conventional (A) and floating (B) systems with the application of cow manure.

and the floating system a diameter of 15.66 mm. The high bulb diameter in the floating culture was greatly supported by the conditions of the growing media, where during the growth stages the rainfall continued to decline, so the media in floating culture was not saturated with water and oxygen was still available. Nutrient absorption, water uptake and root respiration are all affected by oxygen availability, which is a crucial component for plant growth. The use of biological fertilizers or a combination of biological and organic fertilizers can increase the shallot bulb diameter compared to controls (Neira *et al*., 2015; Xiong *et al*., 2015; Purba *et al.*, 2020; Widyastuti *et al.*, 2021).

Shallot production is highly dependent on the weight of fresh and air-dried bulbs. The floating cultivation system yielded the greatest average data, which was significantly different from conventional cultivation (42.33±3.52 g *vs*. 35.69±2.94 g (Table 4). According to Jaya *et al.* (2019), the availability of water below the plant media in the floating system significantly promotes plant growth and yield. Based on the results, conventional farming required a higher dosage of cow manure (20 ton/ha) compared to floating farming, which required only 15 ton/ha. In terms of cost and bulb production, Paputri *et al*. (2016) found that a cow manure dose of 20 ton/ha was economically feasible. However, Arzad *et al.* (2017) found that mustard plants needed up to 25 ton/ha of cow manure.

The estimated production per hectare was calculated using data on air-dried bulb weight under the assumption of a planting space of 20 cm x 20 cm. The highest estimated production was obtained in the floating system from P_2 treatment with 21.61 ton/ha, while the highest production in the conventional system was from P_3 treatment with 12.61 ton/ha. The P_0 treatment had the lowest estimated yield, at 4.38 and 5.43 ton/ha in the conventional and floating cultivation systems, respectively (Fig. 5).

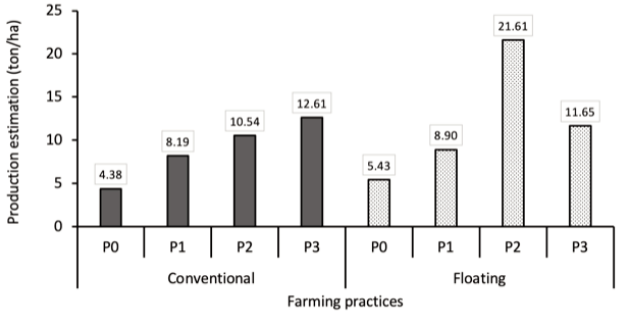

Fig. 5 - Estimated production of shallot from different farming practices with the application of cow manure.

Based on the results, it was concluded that the Bima Brebes shallot variety has the potential to be cultivated using the floating technique with the application of 15 ton/ha cow manure, as seen from production estimation reaching 21.61 tons of dry bulb, which is higher than the 9.9 ton/ha of dry bulb reported by the Ministry of Agriculture (2019). Based on this large potential yield, further research has been carried out again in 2023 to obtain more accurate production data. The results obtained will be the basis for considering recommendations for floating shallot development.

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"Hurdley technologies" utilized to improve postharvest life of asparagus spears (*Asparagus officinalis* L.)

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Key words: *Asparagus officinalis*, metabolic heat, postharvest quality, respiration, UV-C and Gamma irradiation, water status.

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Abstract: Asparagus (*Asparagus officinalis* L.) has short shelf-life due to the high metabolic activity of the apical meristems. Storage at low temperature and high relative humidity is used commercially to keep fresh asparagus spears. Techniques denominated "Hurdley technologies" (UV-C or gamma irradiation) have been tested in fruits and vegetables to extend postharvest life. These technologies were used to extend postharvest shelf life of asparagus spears by inhibition of meristematic activity. Spears were irradiated with UV-C at dosages of 2.46, and 4.93 kJ m^{-2} and gamma irradiation at 1 and 1.5 kGy, before storage at 2°C and 90% relative humidity (RH) for 20 days. Metabolic heat (R_q) was measured in apical meristems, as well as whole spear respiration, sugars content, water potential components and color descriptors. Metabolic heat and whole spears respiration rate did not show differences due to effect of UV-C treatments, while spears treated with gamma radiation showed a metabolic activity inhibition of 10 and 15% for 1 and 1.5 kGy, respectively, while whole spear respiration rate was not affected. Changes in color variables showed a slight reduction in gloss. Sugars content in UV-C remained unchanged, while gamma radiation induced a reduction in glucose. An increase in fresh weight loss was noticeable on those treated with gamma irradiation. No changes in water potential components were observed. It was concluded the treatments used did not reported positive benefits in extending asparagus spears shelf life.

1. Introduction

Green asparagus is a high value vegetable, and its consumption dates back to ancient times (Anido and Cointry, 2008). Worldwide demand is increasing because its gourmet features (Pegiou *et al.,* 2020) and

nutraceutical properties (Fan *et al*., 2015).

This produce is harvested while actively growing and continues to grow even after harvest, keeping a high metabolic activity characteristic of apical meristems (Aegerter *et al.,* 2011). Spears are exposed to different stresses after harvest, such as wounding, dehydration and limited nutrient supply. As a result, they cannot maintain metabolic homeostasis because of their high levels of metabolic activity, respiration rate, and carbohydrate consumption. Consequently, spears metabolites are rapidly consumed, affecting their quality and accelerating senescence during storage, transport, and retailing (Anastasiadi *et al.,* 2020).

Quality loss is mainly noticed by color changes, such as the loss of a bright green color, besides tipping, bending and feathering, which together decreases postharvest quality (Anastasiadi *et al.,* 2020). Concomitantly, dehydration adversely affects fresh weight and turgor loss (Gardea *et al.,* 2023). Low temperatures and high humidity are the conditions commonly utilized in many horticultural products to control senescence and increase shelf life (Singh *et al.*, 2014). However, other methods for fresh produce preservation known as "Hurdley technologies" are available now (Arvanitoyannis *et al.*, 2009), including cold plasma (Jia *et al.*, 2022), UV-C irradiation (Haro-Maza and Guerrero-Beltrán, 2013) and gamma ray irradiation (Prakash and Ornelas-Paz, 2019).

Irradiation based technologies take advantage of plants natural defense capabilities by stimulating plant defense mechanism, including biosynthesis of secondary plant metabolites, as well as defense-related enzymes activation (Ghaouth *et al.,* 2003; Arvanitoyannis *et al.,* 2009; Chipurura and Muchuweti, 2010; Bisht *et al.,* 2021).

Furthermore, it has been demonstrated that UV-C irradiation, both in pre- and post-harvest can inhibit the growth and sprouting of vegetables (Duarte-Sierra *et al.,* 2020; Sinha and Häder, 2002; Verdes-Teodor *et al.,* 2019). The same effect has been observed in some gamma-irradiated vegetables as like potato (Blessington *et al.,* 2015; Mani and Hannachi, 2015), seeds and sprouts (Rajkowski and Latiful, 2012).

UV-C irradiation could induce photomorphogenic responses that encourage growth and development in a distinct way, reaching photoreceptors that utilize signals to activate similar gene expression, and thus, inducing phenotypic changes, such as flowering and stem branching (Darras *et al.,* 2012).

Additionally, gamma irradiation has the potential to destroy DNA, stopping plant meristematic activity (Arvanitoyannis *et al*., 2009), and therefore, they can inhibit potato sprouting (Cools *et al.,* 2014) or reduce growth rate in *Fressia hybrid* L. (Darras *et al.,* 2019).

In summary, reduction in spear tip meristematic activity will reduce respiratory and sugar consumption rates, which in turn will cause a reduction in deterioration rate (Verlinden, 2014). To our knowledge, the effects of ionizing and non-ionizing irradiations on asparagus spear shelf life has not been explored.

Microcalorimetry capacity to measure the heat product of metabolism such as changes in respiratory variables can contribute to our understanding of plants metabolic activity in response to external stimulus (Wadsó and Hansen, 2015). Microcalorimetry is an excellent nondestructive tool for detecting small metabolic changes in intact vegetable tissues. Gardea *et al.* (2023) have suggested that calorimetry is a practical tool to detect changes in tip metabolic activity related to physiological and quality changes that determine spear shelf-life.

Based on the above mentioned, our objective was to evaluate the potential to increase green spears shelf-life by using "Hurdley technologies", such as UV-C and Gamma irradiations.

2. Materials and Methods

Plant material and stored treatments

Asparagus (*Asparagus officinalis* L.) cv. Brock was harvested from a commercial asparagus orchard near Caborca, Sonora, Mexico, 30°47'22.32"N, 112°45'51.98"O. Spears were of standard quality with dimensions of 18 cm in length and 1.0-1.5 cm in diameter. Asparagus were grouped for experiments with non-ionizing and ionizing irradiation. In each, there were 6 groups of 25 spears each and packaged upright into a commercial plastic box (30 cm height x 35 cm length x 30 cm depth) including moisture pads at the bottom.

In the first experiment (experiment 1), asparagus spears were exposed to UV-C irradiation, removed from the top cover of the boxes, and placed inside a laminar hood. The UV-C irradiation was carried out using two lamps (unfiltered General Electric 15 W G15T8 lamps), 15 cm above the box. Time exposure was equivalent to 2.46 and 4.96 kJ m^2 of energy level.

During the second experiment (experiment 2), gamma radiation was applied at absorbed doses of 1 and 1.5 kGy using a Gammacell 2200 Excel irradiator (MDS Nordion, March Road, Ottawa, Notary, Canada) equipped with a ^{60}Co source of activity of 14.4 KCi, and a chamber for irradiating samples with a maximum volume of 3.7 L, located at the University of Sonora, Hermosillo, Sonora, Mexico.

After treatments exposure, spears were stored in a controlled temperature chamber kept at 2°C and 90-95% RH. In each experiment, a non-irradiated group was included as control. Every 4 days (d) tip metabolic activity (R_q), and the respiration rate of whole spear were assessed; also fresh weight loss, water potential turgor pressure and osmotic potential, sugar content (sucrose, glucose, fructose), color lightness (L*), hue angle (H°), chroma (C), and change color ($^{\Delta}E$).

Metabolic heat [R_q, ($\mu W mg^{-1}$)] of asparagus spears apical sections

Microcalorimetry ability to measure changes in metabolic heat can contribute to our understanding of plants metabolic activity in response to external stimulus. Six asparagus spears were sampled for treatment and kept protected in a cooler with moist absorbent paper and ice pack gels to prevent dehydration and transferred immediately to the lab.

Metabolic heat production [R_q, W mg^{-1} of dry weight (dw)] was determined by isothermal microcalorimetry at 25°C, according to Millan-Soto *et al.* (2019). We used a multi-cell differential scanning microcalorimeter CSC 4100 (Calorimetry Science Corporation, Pleasent Grove, Utah, USA), equipped with four sealed metal cells of 1 mL. The fourth cell was used as reference and kept empty during measurements. With the aim to prevent condensation inside the instrument, we kept a steady flow of 1.75 $mLmin^{-1}$ with N_2. The temperature in the instrument chamber was kept constant at 15°C using a circulating-cooling bath (PolyScience, Niles, Illiinois, USA). Three samples were measured simultaneously. In total, six asparagus spears tissues with an average fresh weight of 90 mg were used. To prevent sample dehydration during data acquisition, 50 µL of sterile water were added on each cell and one apical meristem was used per cell, with cut side (basal) in contact with water. After placing the samples, the instrument was allowed to stabilize for about 2500 s to achieve a steady-state metabolic heat rate, which usually happened during the last quarter of this protocol period.

$$CO_2\ (g) + 2OH\text{-}(aq) = CO_3^{2-}(aq) + H_2O\ (aq)\ \Delta H = -108.5\ kJ\ mol^{-1}$$ (Orozco *et al.,* 2022)

Samples were dried in a vacuum oven precision (model 19, Thermo Electron Corp. USA) at 65°C for 48 h. Further, the data were baseline adjusted and calculated based on dry weight.

Whole spears respiration

Carbon dioxide production was determined according to Badillo and Segura-Ponce (2020). Six asparagus spears were placed in a 1 L container (three containers by treatment). Air samples (1 mL) were taken from each headspace after incubation for 1 h and injected into a Varian 3400cx gas chromatograph equipped with a HayeSep N column (2 m x 3.17 mm inside diameter, Supelco, Inc.) coupled to a thermal conductivity detector. The parameters were: 100°C injection temperature, 170°C thermal conductivity detector, and 120°C flame ionization detector. Nitrogen was used as a carrier gas with a flow rate of 25 mL min^{-1} CO_2 production was calculated using CO_2 standards of known concentration according to the algorithm:

$$mL\ CO_2\ Kg^{-1}\ h^{-1} = (spa * Stc * hs) / (Stpa * w * t)$$

Where spa is the sample peak area, Stc is the concentration of the reference standard (CO_2: 0.05 mL L^{-1}), hs is the headspace volume (L), Stpa is the standard peak area, w is the sample weight (kg), and t is the incubation time in hours. Results were expressed in mL CO_2 kg^{-1} h^{-1}.

Sugars

Glucose, fructose, and sucrose were quantified according to Ma *et al.* (2014) with some modifications. Briefly, 10 g of tissue were mixed with 50 mL of distilled water and homogenized for 2 minutes with a T25 Ultra-Turrax homogenizer (IKA, Staufen, Germany) equipped with a dispersing tool (IKA, Staufen, Germany). The samples were filtered, and 2 mL of filtrate were placed in eppendorf vials and centrifuged at room temperature for 15 minutes at 3700 *g*. The supernatant was purified with GV filters with 0.22 µm pore size. 20 µL of sample filtrate were injected into an HPLC (Varian ProStar 210) equipped with a LC-NH_2 column (Sigma Chemical Co., St. Louis; 250 m length, 4.6 mm of internal diameter, and 5 µm of particle size) with an LC-NH_2 guard column and a refractive index detector (Varian ProStar 350). The

mobile phase used was acetonitrile:water (80:20, v/v) at a flow of 1 ml min^{-1}. Quantification was carried out with a mix of standards curves done by injecting into the HPLC equipment solutions of glucose, fructose, and sucrose with different concentrations. The r^2 for glucose, fructose, and sucrose standard curves were 0.9544 and 0.9634, and 0.9321, respectively. Individual sugars were reported as mg g fw^{-1} of sample.

Water status

A Sartorius balance was used to determine spears fresh weight loss (FWL) every four days. Five bunches per treatment were taken and their initial and final weights (at the end of the storage period) were recorded. The results were averaged and expressed in percentages of FWL.

In the middle segment of the spear, a cylinder 1.5 cm long was cut to evaluate water potential (Ψw). Three asparagus spears were used for treatment every 4 d, according to Muy-Rangel *et al.* (2004). Three spear middle segments were weighed and soaked in sucrose solutions with molalities of 0.1, 0.05, 0.015 and 0.025.

The osmotic potential (Ψs) component was calculated by the equation Ψs = -CiRT; where Ci is sucrose molality, R is the gases ideal constant (0.0083 $Kg{\cdot}MPa{\cdot}mol^{-1}\,K^{-1}$) and T is the temperature at 273 °K (Rodríguez-Burgos *et al.,* 2015). Sucrose solutions pressure potential (Ψp) is zero because the solution is not inside of a container. Pressure potential was calculated from the equation Ψw = Ψp – Ψs, taking into account that pressure potential is zero, we concluded that osmotic potential equals tissue water potential Ψw = -Ψs (Rodríguez-Burgos *et al.,* 2015). Samples were left to rest for 2.25 h (time required to reach equilibrium between samples and solution). Then removed and excess water dried on tissue paper and their weights were recorded. After that, sucrose solution, in which the tissues did not gain or lose weight, corresponds to water potential.

The Ψs was determined in the middle segment of 3 spears with a steam pressure osmometer Wescor model 5520 using the methodology developed by Jia *et al.* (2020). A sap sample obtained from the middle segment was preserved at -20°C until use. After defrosting at room temperature, 10 µL of exudate was placed on a 0.32 cm^2 filter paper disc and placed inside the chamber. The osmometer was calibrated with standards of 100, 290, and 1000 mg kg^{-1} NaCl solution and the results of molality were converted to an osmotic potential with the Van't Hoff equation, where Ψs = CiRT (Jia *et al.,* 2020). Pressure potential (Ψp) was calculated with the difference between osmotic and water potentials according to Jia *et al.* (2020).

Color

The color of spears was measured with a portable colorimeter (Minolta CR200, Konica Minolta, Japan). The L*, a*, and b* color space variables were recorded in the tip, middle and basal segments of 10 asparagus spears, every four days. Differential color parameter ($^{\Delta}E$) was calculated using the following equation: $(^{\Delta}E) = [(L^*-L^{*0})^2+ (a^*-a^{*0})^2+ (b^*-b^{*0})^2]^{1/2}$; where: L^{*0}, a^{*0}, and b^{*0} were the readings of the color space at zero-day; while L*, a*, and b* correspond with the color space variables determined at each sampling day (Kohli *et al.,* 2022). Hue angle (H°) was calculated from a* and b* color space variables using the following equation: [H°=tan-1 (b*/a*) when a*>0 and b*>0 or H°=180+ tan-1 (b*/a*) when a*<0 and b*>0)], and saturation color named chroma using the following equation: $C = (a^{*2}+b^{*2})^{1/2}$ (Kohli *et al.,* 2022).

Statistical analysis

Analysis of variance (ANOVA) was performed based on a completely randomized design, with a factorial arrangement of treatments, where two irradiation protocols, plus controls were established and evaluated on six dates (3 x 6). When main effects significant differences (p<0.05) were found, Tukey multiple range tests were carried out. All the analysis were done with the Infostat 2017e version (University of Cordoba, Cordoba, Argentina).

3. Results and Discussion

Metabolic heat of meristematic apical tissue and respiration rate of whole pears.

Statistical analysis did not find a significant interaction between storage period and UV-C dosages. UV-C irradiated spears showed a metabolic heat (*Rq*) significant reduction during storage at 2°C ($p<0.05$) (Table 1). All samples started at 40 µw mg^{-1} dw, then steadily decreased until reaching 22 µW mg^{-1} dw on day 20. It follows the same pattern as that reported by Gardea-Bejar *et al.* (2023) which found a constant decrease in metabolic heat by spears stored at 2 and 5°C. Regarding UV-C dosages, no significant differ-

Table 1 - Effect of the UV-C radiation over physiology and quality parameters in asparagus during storage at 2°C, independent of level energy

Variable	UV-C					
	Storage time (days)					
	0	4	8	12	16	20
Metabolic heat (µW mg^{-1}dw) **	40.3 e	34.2 d	29.2 c	26.5 bc	23.6 ab	22.4 a
Respiration (mL CO_2 Kg^{-1} h^{-1}) **	15.8 d	12.3 c	10.2 a	10.4 ab	9.7 a	11.1 b
Fructose (mg g^{-1} fw)*	14.24 a	10.48 a	9.95 a	8.72 a	7.94 a	7.60 a
Glucose (mg g^{-1} fw)*	16.22 b	10.95 ab	10.76 ab	8.51 a	8.16 a	7.58 a
Chroma (C)	38.6 a	37.8 a	38.2 a	38.3 a	37.5 a	38.1 a
Hue (°)	114.3 ab	115.0 b	114.7 ab	114.3 ab	113.8 ab	112.9 a
L**	47.7 abc	49.0 c	49.0 c	48.04 bc	46.59 ab	45.58 a
ΔE	0.0 a	3.2 b	3.7 b	3.6 b	4.5 b	4.3 b

*Significant interaction, ** Highly significant interaction.
Means followed by same letters within rows are not statistically significant (Tukey, p > 0.05).

ences were found between controls and UV-C treated spears, averaging of 29 µW mg^{-1} dw.

Gamma-exposed spears data did not report significant interactions, but significant main factors. As far as storage period, no differences (P>0.05) were found in the first eight days averaging ca. 39 µW mg^{-1} dw. However, starting on day 12^{th}, significant reductions (P<0.05) took place decreasing to 10.9 on day 20 (Table 2). As far as gamma irradiated treatments a significant effect was found between controls and 1 and 1.5 kGy averaging values of 34.7, 31.5 and 27.0 kGy (Table 3), respectively. Thus, implying a severe injury by gamma radiation to meristematic cells, as reported elsewhere (Dina *et al.,* 2018).

Usually, deterioration rate of harvested commodities is proportional to their respiration rate (Kader, 1992). A significant reduction, although somehow erratic, in respiration of UV-C treated spears was found along the 20 days storage, starting at 15.8 mL CO_2 Kg^{-1} h^{-1} and eventually leveling to 11.1 units (Table 1). Slightly higher respiration rate was observed in UV-C treated tomatoes with 15.8- and 19.8- mL CO_2 Kg^{-1} h^{-1} at 20°C irradiated with at 0.003 y 0.033 KJm^{-2} (Cote *et al.,* 2013). Even though asparagus apical meristem shows the highest respiratory activity (Anastasiadi *et al.,* 2022) and that is why the respiration rate of the upper segment (apical zone) is the one with the highest rates (Verlinden *et al.,* 2014). Further, the normal behavior for whole asparagus spears in postharvest is the reduction in respiration rate after harvested (Wu and Yang, 2016). In cherry (Michailidis *et al.,* 2019), and broccoli florets (Costa *et al.,* 2006), UV-C irradiation affected respiratory metabolism, resulting in a slower respiratory rate than in non-irradiated plants (Yang *et al.,* 2014).

As far as UV-C treatments, a slight but significant

Table 2 - Effect of the gamma radiation on physiology and quality parameters in asparagus during storage at 2°C, independently of energy level

Variables	Gamma					
	Storage time (days)					
	0	4	8	12	16	20
Metabolic heat (µW mg^{-1}dw) **	38.7 d	40.4 d	38.0 cd	33.0 c	27.2 b	10.9 a
Respiration (mL CO_2 Kg^{-1} h^{-1}) **	15.8 c	10.3 b	10.3 b	10.7 b	4.38 a	4.5 a
Fructose (mg g^{-1} fw)	10.52 a	8.24 a	8.16 a	-----	-----	-----
Glucose (mg g^{-1} fw)*	6.67 a	5.02 a	6.36 a	-----	-----	-----
Chroma C©	34.5 ab	33.9 a	33.2 a	35.8 b	34.6 ab	34.0 a
Hue (°)	118.2 a	118.3 a	118.9 a	115.1 a	115.6 a	114.4 a
L**	47.7 c	38.7 a	41.5 b	41.8 b	41.7 b	41.3 b
ΔE	0.0 a	15.2 e	12.8 d	9.1 b	12.2 d	11.0 c

*Significant interaction, ** Highly significant interaction.
Means followed by same letters within rows are not statistically significant (Tukey, p > 0.05).

change in response to irradiation was found with values of 11.8, 11.3 and 11.5 for controls, irradiation at 2.4 and 4.96 KJm^2, respectively (Table 3). We hypothesized that low respiration rate may be caused by reducing succinic dehydrogenase and cytochrome C oxidase activities and higher integrity of mitochondrial membrane with the consequence of lower permeability and gas exchange, as was demonstrated in UV-C irradiated peach during postharvest (Yang *et al.,* 2014).

Table 3 shows that gamma-irradiated spears went through a statistically significant reductions in respiratory activity during storage starting at 15.8 mL CO_2 Kg^{-1} h^{-1} on day 0 and ending at 4.5 on day 20^{th}. Gamma irradiation dosages induced a significant reduction ($P<0.05$) with increasing irradiation strength with values of 10.8, 8.7 and 8.4 mL CO_2 Kg^{-1} h^{-1} for the 0, 1 and 1.5 kGy treatments (Table 3). As reported in several crops, low dosages resulted in increasing responses (Lu *et al.,* 2023), while exposures above 0.5 kGy caused decreases, (Ali *et al.,* 2015). A clear impact of gamma irradiation on bamboo shoots irradiated at 0.5 kGy and stored at 2°C and 90% RH decreased respiratory rate at 60 and 5 % at the beginning and end of the experiment (Zeng *et al.,* 2015), Preuss and Britta (2003) stated that a high dose of gamma radiations affects cell cycle arrest during G2/M phase, inhibiting growth during cell division, consequently causing a drop in respiratory rate.

Sugar content in asparagus spear

Sucrose was not detected in asparagus spears, while fructose and glucose were present in both experiments.

Constant declines in fructose and glucose contents were observed along storage, although statistically significant only for glucose, starting at 16 mg g^{-1} fw and ending below 7.6 (Table 1). Such behavior has been reported by Davies *et al.* (1996) in spears of the cultivar Limbras with sugar values falling down 30% in the first three days of storage.

Reductions in sugar content is normal for asparagus during postharvest due to their constant energy needs to maintain their high respiratory activity (Sergio *et al.*, 2019).

Same pattern was found in Gamma irradiation spears (Table 2), although this experiment last only eight days of storage. Since gamma irradiation accelerates spear metabolic activity, glucose is consumed rapidly (King *et al.,* 1990), triggering senescence as a result, in addition to depletion caused by damage to tissue membranes and cell walls (Ali *et al.,* 2015).

Water status

Spears fresh weight losses (FWL) did not differ between UV-C irradiated treatments and controls. At 4th and 8th days of storage, FWL accounted for 2 and 3% respectively, on days 12th and 16th, increased to 5 and 7%, and finally at day 20 reached 13% (Fig. 1A). Wang *et al.* (2019) found results slightly lower in spears wrapped in perforated film, reporting a FWL of only 2 % after 20 days in storage at 2°C at 85% RH. Further, Wang *et al.* (2020) reported FWL within a range of 3.0-5.0% in asparagus under modified atmosphere and stored at 4°C.

Gamma irradiated spears showed significant

Table 3 - Effect of different UV-C and Gamma irradiation over physiology and quality parameters in asparagus during storage at 2°C. Values are the average of six sampling dates

Experiments	UV-C (KJ m^{-2})			Gamma (kGy)		
Variable	0 (control)	2.4	4.96	0 (control)	1.0	1.5
Metabolic heat (μW mg^{-1}dw) **	30 a	29 a	29 a	34.7 c	31.5 b	27.9 a
Respiration (mL CO2 Kg^{-1} h^{-1}) **	11.8 b	11.3 a	11.5 ab	10.8 b	8.7 a	8.4 a
Fructose (mg g^{-1} fw)	9.97 a	9.99 a	9.55 a	9.35 a	8.87 a	8.70 a
Glucose (mg g^{-1} fw)*	10.91 a	10.40 a	9.72 a	10.33 b	3.11 a	4.61 a
Chroma (C)	38.9 b	37 a	38.2 ab	35.1 b	33.9 a	34.0 a
Hue (°)	115.1 b	113.7 a	113.7 a	126.4 a	131.7 a	137.3 a
L**	47.88 a	47.92 a	47.05 a	45.8 b	40.7 a	39.8 a
ΔE	2.4 a	2.9 a	4.5 b	5.1 a	11.9 b	13.2 c

* Significant interaction, ** Highly significant interaction.
Means followed by same letters within rows are not statistically significant (Tukey, $p>0.05$).

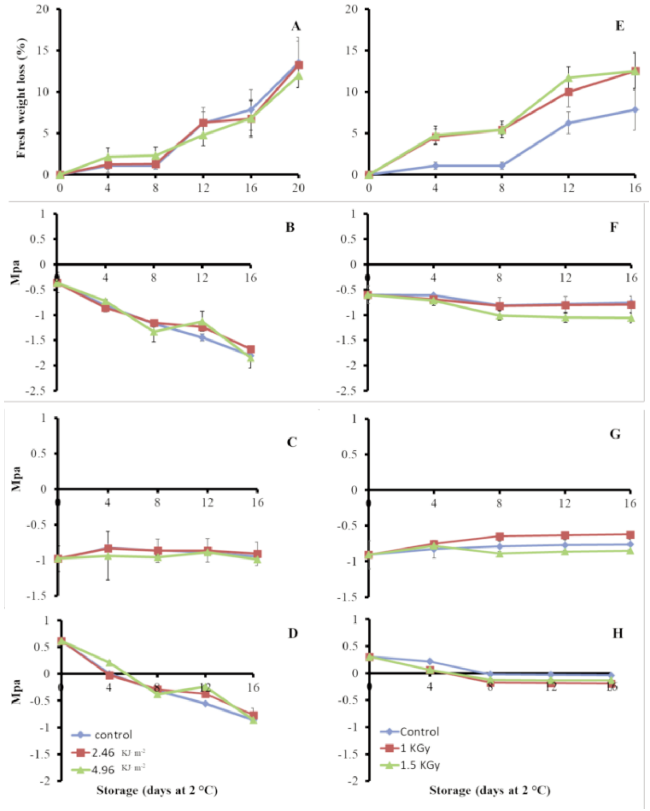

Fig. 1 - Changes in fresh weight loss (A and E), water potential (B and F), osmotic pressure (C and G) and turgor potential (D and H) in asparagus spears cv. Brock treated with UV and Gamma levels as indicated and stored at 2°C and 90% of relative humidity. A, B, C, and D graphics correspond UV-C irradiation experiment; E, F, G and H graphics correspond Gamma irradiation experiment. Differential color parameter (ΔE). Values increased during storage from 0 to 4.3 units at day 20 (Table 1). While the highest UV-C dosage caused almost a double-fold significant ($p<0.05$) increase from 2.4 to 4.5 units (Table 3).

effects on spears FWL. After 16 days of storage, FWL accounted for 12% in the irradiated samples, and only 7.5% in controls (Fig. 1E). By contrast, Lescano *et al.* (1993) found that FWL increased linearly, without significant differences between controls and irradiated samples in white asparagus with doses of 1.0, 1.5 and 2 kGy. These data support the rapid senescence of irradiated spears.

No differences among treatments in water potential for both experiments were found on each sampling day. Ψw decreased gradually, from -0.5 Mpa at 0 days down to -2 Mpa at 16 days in the UV-C experiment (Fig. 1B). Gamma-irradiated asparagus decreased at slightly slow rate down to only -1Mpa (Fig. 1F). Although no significant differences were found within each experiment, UV-C treated spears reached final values around -1.75Mpa, while Gamma irradiation caused a mean decrease between -0.5 and -1.0 Mpa. Therefore, as far as water potential, gamma irradiation caused a less aggressive response. Osmotic pressures (Ψs) remain constant in both experiments without significant changes during the 16 d storage at 2°C (Fig. 1C and G).

Spear turgor potential (Ψp) in UV-C fell below the 0 Mpa threshold at day 4 and kept decreasing down to -4 Mpa on day 16 (Fig. 1D). On the other side, gamma irradiated asparagus remained basically constant at around 0 MPa along the 16 days of storage, subsequently, it was no longer possible to evaluate the water status of the asparagus spears gamma irradiation treated after day 16th (Fig. 1H).

According to Siomos (2003), asparagus spears have between 92 and 94% water content, which reduces drastically once harvested, the loss of commercial quality occurs when the FWL exceeds 8%. Spears in the UV-C treatment reached such threshold only until day 16; although turgor pressure fell below 0 on day 8 without showing any visual dehydration. When samples were Gamma irradiated FWL did exceed 8% on day 12, showing obvious signs of dehydration, a cooked appearance, slippery epidermis, and loss of rigidity because of the effects of gamma irradiation (Jeong and Jeong, 2018).

Color parameters

Table 1 shows the results of C, H°, L* and $^{\Delta}$E in asparagus spears treated with UV-C kept at 2°C for 20 d.

Chroma (C), no significant changes were found during the 20 days storage period. However, UV-C at 2.4 KJ·m^{-2} did cause a small, but significant decrease, as compared to controls and the 4.96 KJ·m^{-2} exposure (Table 3). The C results of both experiments found in this work agree with data published by Costa *et al.* (2006), who found similar tendencies, suggesting the slight presence of a dull color.

In the variable of Hue, erratic but significant changes were observed, although after 20 days of storage showed 113 units, similar to initial values. UV-C radiation at both exposures caused a small and significant reduction from 115 to 113.7 units (Table 1). These values are slightly lower than reported by Wang *et al.* (2020) in asparagus treated with CO_2 and jept under modified atmospheres at 4°C with values of 120-122, corresponding to slightly yellow.

Significant changes were recorded in L, although somehow erratic, increasing on days 4, 8 and 12, following to a decrease to initial values. As far as the

effects by UV-C on L, no significant changes occurred (Table 1).

These slight color changes observed in green fruits and vegetables treated with UV-C are probably due to chlorophyll degradation induced by irradiation damage to the chloroplast and biosynthesis of some anthocyanins (Siomos *et al.,* 2000; Villanueva *et al.,* 2005; Li *et al.;* 2006). Indeed, in our lab, we investigated the development of yellowish color in cucumbers treated with large UV-C irradiation energy levels and observed a color change from intense green to a paler green (data not shown). However, in the UV-C irradiation experiment, the differences in color observed in this experiment probably are enough to cause a significant change as to induce consumer rejection.

Table 3 shows color-related results of Gamma-irradiated spears at two intensities. A significant, but erratic, response was observed in Chroma during storage ending with values similar to the initial 35.5 units. Gamma irradiation exposures caused a significant, although small, reduction of 1.1 units in C (Table 2).

Gamma irradiation did not cause significant changes in Hue during the 20 days storage with values averaging 114.4 units. (Table 2).

Gamma irradiation did cause a significant and overall reduction in L during storage, ending on 41.3 units as compared to the initial 47.7 (Table 2). Also, significant reductions were found when exposed to two gamma irradiations, with 40.7 and 39.8 units for 1 and 1.5 kGy, respectively (Table 3). Gamma irradiated papayas at 0.75 kGy stored at 11°C and 90 % RH showed similar behavior. Indeed, irradiated papaya (C = 40.89 and L* = 51.50) achieved a slightly more intense color, as compared with non-irradiated controls (C = 41.67 and L* = 52.50). Borzouei *et al.* (2013) determined that chlorophyll content in cv. Roshan increased after exposure to radiations at 100 kGy, which could correlate with stimulated metabolism of pigment biosynthesis, suggesting that yellow color developed slightly more quickly in controls (Pimentel and Walder, 2004).

Peak color differential ($^{\Delta}$**E)** was recorded just after 4 d in storage reaching 15.2 units, followed by significant decreases reaching 11 units at day 20 (Table 2). Exposure to gamma irradiation did cause a significant increase in this variable with controls showing 5.1 units and 11.9 and 13.2 units for exposures at 1 and 1.5 kGy irradiation causing a loss of glossy appearance (Table 3). Such change in the green chlorophyll appearance from bright to dull is associated with decrease of L and slight changes in chroma may result from deformations in thylacoyd structure and damage to chloroplasts (Wi *et al.,* 2007; Choi *et al.,* 2021).

4. Conclusions

"Hurdley technologies" do not extend postharvest shelf life in asparagus spears cv. "Brock", although the UV-C irradiation with energy levels of 2.46 $kJ.m^{-2}$ and 4.92 $kJ.m^{-2}$ did not adversely affect spear postharvest quality. The levels of UV-C radiation applied did not affect metabolic activity significantly. A slight tendency to develop a yellowish coloration was observed when irradiated with UV-C 4.96 KJm^{-2}.

Gamma irradiated spears decayed rapidly, as demonstrated by several variables. Such as a drastic decrease in metabolic heat production and respiration, as well as development of a glossy color, cooked appearance, slippery epidermis, and losses in fresh weight and turgency. At the intensities tested, Hudley technologies do not extend postharvest shelf-life of asparagus spears.

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A biostimulant complex comprising molasses, *Aloe vera* extract, and fish-hydrolysate enhances yield, aroma, and functional food value of strawberry fruit

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Abbreviations: A = achromatic; ATC = automatic temperature compensation; B = blue; BC = Biostimulant complex; C = cyan; EC = electrical conductivity; F-C = Folin-Ciocalteu; FTIR = Fourier transformed infrared; G = green; GAE = gallic acid equivalent; GLM = general linear model; GM = genetic modification; M-IR = mid-infrared; O = orange; PCA = principle component analysis; Pi = pink; PLS-DA = partial least squares-discriminant analysis; Pu = purple; QE = quercetin equivalent; R = red; RWC = relative water content; sPLS-DA = sparse partial least squares-discriminant analysis; SSC = soluble solids content; TP = total phenolics; UATR = universal attenuated total reflectance; W = white; Y = yellow.

Abstract: Strawberry is a popular functional food due to the presence of antioxidant and anti-inflammatory phytochemicals. Enhancing this functional food value is an opportunity to improve consumer health, but strategies to do so cannot compromise yield or organoleptic properties, which are highest priorities for farmers and consumer, respectively. One promising strategy is the supplementation of fertiliser regimens with biostimulants, which are non-nutritive substances associated with species-specific improvements to crop growth, yield, and quality. Accordingly, the impacts of a biostimulant complex (BC) containing molasses, *Aloe vera* extract, and fish-hydrolysate is characterised herein for its potential to impact strawberry growth, yield, quality, and functional food value. Results indicated that BC treatment significantly increased ($p < 0.05$) plant biomass and canopy area (growth), total fruit count and weight per plant (yield), fruit aroma and colour (quality), and antioxidant potential (functional food value). The results presented highlight the potential utility of biostimulants to the strawberry sphere, providing a strategy to enhance the fruit to the benefit of both farmers and consumers.

1. Introduction

The genus *Fragaria* is comprised of 25 species of small flowering plants known as strawberries, which are widely cultivated for their edible fruits (Hirakawa *et al.*, 2014). Of these 25 species, the hybrid octoploid *Fragaria* x *ananassa*, is the most popular variety, accounting for 60% of the worlds strawberry fruit production (Amil-Ruiz *et al.*, 2011). The global strawberry industry is a profitable and growing industry, with world production outputs in 2020 equal to 40.76 million tonnes (FAO, 2022). Whilst strawberry fruits are primarily consumed as a fresh fruit, they are also popular additions to processed foods such jams, juices, dairy products, and flavoured drinks, making strawberries one of the most popular and versatile global agricultural products (Moraga *et al.*, 2006; Basu *et al.*, 2014).

The drivers of consumer perception of strawberry fruit quality are their physical features, organoleptic properties, nutritional value, and added secondary health benefits (functional food properties) such as antioxidant, anti-inflammatory, and anti-hypertensive activities (Basu *et al.*, 2014). Improving these measures is therefore desirable to consumers who would experience higher quality produce and potentially added health benefits from increased functional food properties. The antioxidant activity of strawberry fruits is a particular driver of the fruit's popularity, as this functional food property is associated with a range of benefits including improvements to cardiovascular health (Giampieri *et al.*, 2015), neurodegeneration (Esposito *et al.*, 2002), cancer (Zhang *et al.*, 2008), and type 2 diabetes (da Silva Pinto *et al.*, 2010). The antioxidant activity of the fruit is attributed to the high concentrations of polyphenols including flavonoids, anthocyanins, and ellagitannins, and vitamins such as ascorbic and folic acid, which have been shown to vary in concentration in fruits depending on cultivar, storage, processing, and cultivation system (Basu *et al.*, 2014; Afrin *et al.*, 2016). In addition to improving the functional properties of the fruit, strawberry yield and organoleptic improvements are also significant opportunities to benefit the strawberry industry.

Processing (Moraga *et al.*, 2006), plant breeding (Diamanti *et al.*, 2012), and cultivation practises (Akhatou *et al.*, 2014) have all been shown to impact fruit sensory properties such as firmness and taste, however, breeding is slow and costly, and processing is unsuitable to the fresh fruit sector. Therefore, modifications to farming practises could be potentially fast and versatile interventions to strawberry cultivation, to improve fruit quality metrics - functional food potential and organoleptic desirability - and thereby enhance customer perception of quality and value returned to farmers. Accordingly, implementation of biostimulants continues to generate interest as a fast and environmentally friendly improvement to cultivation practises, as these inputs are non-nutritive compounds or substances which beneficially impact plant growth, development, or yield (Du Jardin, 2015). Various biostimulants have been associated with improvements to fruit quality in a range of species, however effects are plant-species and dosage dependent, requiring further exploration of these effects in target crops such as strawberry (Rodrigues *et al.*, 2020; Wise *et al.*, 2020).

Molasses is a by-product from the sugar industry, which is rich in simple and complex carbohydrates, proteins, amino acids, organic acids, and Maillard reaction by-products (melanoidins) (Najafpour and Shan, 2003; Chandra *et al.*, 2008). It has been used as an additive in agriculture and has demonstrated biostimulant effects including increasing yield in beetroot (Nadeeka and Seran, 2020), sugar cane (Srivastava *et al.*, 2012) and spinach (Pyakurel *et al.*, 2019), improved biomass growth in maize (Shahzad *et al.*, 2018) and sorghum (Suliasih and Widawati, 2016), enhanced salinity tolerance in thyme (Koźmińska *et al.*, 2021), and improvements to disease resistance in various species (Welbaum *et al.*, 2004). Similarly, molasses distillery effluent, which is more dilute but similarly comprised to molasses, also has demonstrated biostimulant benefits such as increased yield in banana (Thakare *et al.*, 2013) and sweet pepper (Gaafar *et al.*, 2019), increased nutrient uptake in radish (Hatano *et al.*, 2016), improved growth and development of rapeseed (Li *et al.*, 2020), altered antioxidant activity in cabbage (Bimova and Pokluda, 2009) and black bean (Elayaraj, 2014), as well as increased heavy metal uptake by common reed and sedge for potential applications to bioremediation (Nagy *et al.*, 2020). Accordingly, utilisation of molasses as a biostimulant input for strawberry farming may be expected to positively impact a range of yield, growth, and quality measures.

Aloe vera extracts are also complex mixtures containing plant nutrients, vitamins, enzymes, amino acids, sugars, hormones, and hormone-like compounds (Ishartati *et al.*, 2019; Cortés *et al.*, 2021). The biostimulant effects associated with application

of *Aloe vera* extracts are varied, including increased propagation efficiency and growth of Populus tree clones (El Sherif, 2017), eucalyptus tissue cultures (Hendi, 2021), and grape vine cuttings (Uddin *et al.*, 2020), improved growth, biomass, and oil content of sweet basil (Hamouda *et al.*, 2012), increased growth, yield, oil content, and nutritional content of caraway (Khater *et al.*, 2020), improved growth and chlorophyl content of fenugreek (Al-Yasiri *et al.*, 2021), increased leaf growth and terpene content in lavender (El Sherif *et al.*, 2020), enhanced fruit yield and nutritional content of okra (Hemalatha *et al.*, 2018 a, b), and dose-dependent positive and negative effects on cereal germination (Baličević *et al.*, 2018). Accordingly, *Aloe vera* extracts are utilised as fertiliser additives to improve plant growth and have the potential to improve a range of attributes when added to strawberry plants during cultivation.

Agricultural amino acids can be purified or complex mixtures, which are often extracted (hydrolysed) from animal, plant, or microbial products (Calvo *et al.*, 2014). Legume-derived hydrolysates are common sources of amino acids and have a range of beneficial effects associated with their use during cultivation including increased vegetative growth, yield, and secondary metabolite production in capsicum (Ertani *et al.*, 2014), and increased yield (Colla *et al.*, 2017), nutritional content (Colla *et al.*, 2017; Rouphael *et al.*, 2017), firmness (organoleptic property) (Mirabella *et al.*, 2021), and antioxidant activity (Caruso *et al.*, 2019) in tomato. Furthermore, amino acids derived from fish-hydrolysate have demonstrated increased yield in tomato (García-Santiago *et al.*, 2021), pig blood-hydrolysate increased phenolic and antioxidant properties of lettuce (Zhou *et al.*, 2022), and a commercial amino acid product was shown to increase antioxidant activity in the leaves of *Aloe vera* (Ardebili *et al.*, 2012). Amino acids are also associated with improved nutrient uptake in plants, either directly through provision of organic-N or indirectly through stimulation of soil microbes or chelation of nutrients (Callahan *et al.*, 2007; Halpern *et al.*, 2015). Accordingly, addition of amino acids during strawberry cultivation may demonstrate biostimulant effects to improve a range of measures and thereby benefit consumers and/ or farmers.

Noting the potential benefits of molasses, *Aloe vera* extract, and fish-hydrolysate as biostimulants during strawberry cultivation, the aim of this study was to explore impacts to strawberry growth, yield, and quality associated with the supply of a complex of these biostimulants. This involved the hydroponic growth of strawberry plants with application of the biostimulant complex, followed by temporal assessment to impacts to fruit yield, in addition to end point fruit quality measures such as fruit antioxidant potential, and fruit sensory profile. Characterisation of the effect of these biostimulants to strawberry has the potential to benefit both strawberry farmers and consumers by providing a cost efficient and easily implemented farming strategy to enhance the value of strawberry yields.

2. Materials and Methods

Plant materials and growth conditions

Stock tubes of strawberry plants (*Fragaria* x *ananassa* 'Albion'), supplied by Sunny Ridge Strawberry Farm Pty Ltd. (Boneo, VIC, Australia), were planted into 15 cm pots in Coco perlite substrate (Nutrifield Pty Ltd., Melbourne, VIC, Australia) and maintained in indoor growth rooms for 5 weeks with Coco A&B nutrients (Nutrifield Pty Ltd., Melbourne, VIC, Australia) at pH = 5.8, and EC = 1.0. Fertigation was delivered via a flood and drain system, wherein trays containing potted plants were filled with fertigation liquid to 75% the height of the pots and subsequently drained. Plants were grown under 315W ceramic metal-halide horticultural lamps (315W CMH Pro 4200K, Indoor Sun, Melbourne, VIC, Australia), with a Recom 315W Ballast (Lucius, Melbourne, VIC, Australia), and light:dark (L:D) photoperiod of 12 hours day and 12 hours night. Environmental conditions were restricted to day temperature and relative humidity of 21.5°C, and 70%, respectively, and night temperature and relative humidity of 18°C, and 51%, respectively. After 5 weeks, plants were separated into treatment groups and re-potted into 30 cm square pots with Coco perlite substrate and a top layer (2 cm) of Hydro Clay (Nutrifield Pty Ltd., Melbourne, VIC, Australia).

Treatment and fertigation programme

Twelve plants were split into 2 groups (n = 6): control group, receiving Coco A&B nutrients as per usage instructions, and treatment group, receiving Coco A&B nutrients as per usage instructions plus the biostimulant complex (BC) comprising molasses (10% w v^{-1}), *Aloe vera* extract (2.5% v v^{-1}), and fish-hydrolysate (5% v v^{-1}) at 2 mL L^{-1} during weeks 10-18. Fertigation was delivered to plants via a recirculating drip-irriga-

tion system (4 × 4 L h^{-1} $dripper^{-1}$ $plant^{-1}$) as described in Table 1. The elemental composition, phytohormone profile, and metabolite profile of the biostimulant complex is provided in supplemental tables S1, S2, and S3, respectively.

Table 1 - Fertigation programme for strawberry plants during treatment (weeks 6-18)

Weeks	Fertigation programme (split evenly throughout the day)
6-11	3 × 10 min
12-13	4 × 10 min
14-18	6 × 10 min

Vegetative measurements

Leaf colour (*L*a*b**) was measured using a CR-400 Chroma Meter colourimeter (Konica Minolta, Tokyo, Japan). Canopy area was measured using the smartphone application Easy Leaf Area Free (Easlon *et al.*, 2014). At the conclusion of the harvest period leaf and crown number were counted, and final fresh- and dry-weight measurements were taken for leaf and non-leaf tissues. Vegetative tissues were dried in an ED 53 oven (BINDER, Tuttlingen, Germany) at 70°C for 3 days.

Harvesting and fruit measures

Ripe fruit - defined as BBCH = 87 according to Wise *et al.* (2022) - were harvested, immediately measured (weight and length), and placed into a DT5600 Food Dehydrator (Sunbeam, FL, USA) at 55°C for 7 days. Strawberry fruit length at harvest was measured as the perpendicular distance from the centre of the calyx to the tip of the receptacle. Fruit width at harvest was measured by image analysis using ImageJ (Schneider *et al.*, 2012). In short, image global pixel scale was set based on known fruit length and the 'measure' feature within the ROI manager was utilised to measure the widest fruit diameter.

Determination of pH and Brix of crude fruit extract

A crude extract was prepared by pressing fresh strawberry fruits through four layers of muslin cloth and subsequently passing the filtrate through an additional single layer of muslin cloth. An automatic temperature compensation (ATC) portable refractometer (Sugar/Brix Refractometer 0-32% 300001, Super Scientific Ltd., Scottsdale, AZ, USA) was used to measure Brix (°Bx) of the pure crude extract. A 1/1000 dilution of the crude extract in water was used to measure pH (Sension+ MM 374 GLP 2 channel Laboratory Meter with Sension + 5014T pH liquid combination electrode with silver ion barrier, HACH, Loveland, CO, USA).

Fruit phytochemical extraction

Whole fruit extraction was carried out with adaptations to the method described in Chandra *et al.* (2014). In short, the dried strawberry fruits were pulverised in a 'multigrinder II' (Sunbeam, FL, USA) and then extracted in 8 mL ethanol (100%) per g pulverised fruit. The extraction was carried out in a sonicator at 40°C for 10 min and then filtered through 7-10 µm membrane filter paper with 0.1% ash content (Westlab, Ballarat, VIC, Australia). The ethanol filtrate was evaporated in a 100°C water bath to achieve a dried extract, which was then resolubilised in 5 mL 5% methanol in a sonicator at 40°C for 10 min.

Determination of total phenolic and flavonoid content

Total phenolic (TP) and flavonoid content was determined as per the Folin-Ciocalteu (F-C) method, and the aluminium chloride colorimetric method, respectively, which were adapted from those described in Chandra *et al.* (2014). In short, TP content was determined by combining 100 µL of resolubilised extract with 100 µL F-C reagent, 300 µL 8% w v^{-1} saturated sodium carbonate solution, and 1.5 mL distilled water. Solutions were reacted under light in a PS-10A sonicator (Jeken, Dongguan, China) at 40°C for 30 min. The absorbance was measured at 765 nm and phenolic content was calculated as gallic acid equivalent per gram dry fruit (GAE g g^{-1} d.w.). Total flavonoid content was determined by combining 1 mL of the resolubilised extract with 1 mL 10% (w v^{-1}) aluminium chloride. The solutions were reacted at room temperature for 1 h, and then absorbance was measured at 420 nm. The results were calculated as quercetin equivalent per gram dry fruit (QE mg g^{-1} d.w.). All absorbance measurements were analysed using the DR 5000™ UV-Vis Spectrophotometer (HACH, Loveland, CO, USA).

Sensory perception testing

A blinded test was conducted to explore if the biostimulant treatment was associated with changes to sensory perception of fruits. Participants (n = 6) were asked to score the fruits (on a 9-point scale)

based on their texture, taste, and aroma (Table 2).

Plant image colour analysis

Colour data of strawberry fruit images was extracted as per Wise *et al.* (2022), wherein individual pixels were categorised as either achromatic (A, light grey-black), blue (B), cyan (C), green (G), orange (O), pink (Pi), purple (Pu), red (R), white (W), or yellow (Y), based on maximal similarity to predefined colours.

Mid-infrared (M-IR) analysis

Infrared spectra of the dried strawberry fruits were collected using a Spectrum 2 FTIR spectrophotometer (PerkinElmer Inc., Waltham, MA, USA) equipped with a Universal Attenuated Total Reflectance (UATR) accessory with diamond crystal. Spectra were collected with a 4 cm^{-1} resolution over the 500-7000 cm^{-1} range with four accumulations to produce an averaged spectrum. For data analysis the 4000-7000 cm^{-1} range was excluded. Spectral data were standardised to 1875 cm^{-1} prior to analysis, corresponding with the region of the sample spectra which has minimal influence from the presence of water as indicated by the water absorption spectra (NIST, 2022), presented in the NIST Chemistry WebBook (Linstrom and Mallard, 2001).

Statistical analysis

Analyses implemented during exploration of treatment effects on individual measures included general linear model (GLM), Tukey's test 95% confidence grouping analyses, Anderson-Darling normality test, and Mood's median test in the Minitab 19 statistical software package (Minitab Inc., State College, PA, USA). Analyses implemented to explore treatment effects on the profiles of plant measures (and fruit M-IR spectra) were heatmap analysis, dendrogram, principle component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), sparse PLS-DA (sPLS-DA), and fold change (1.3-fold threshold), using the web-tool Metaboanalyst 5.0 (Chong *et al.*, 2019). The profiles of plant measures were normalised within Metaboanalyst using the cube root transformation and pareto scaling functions, while M-IR data was transformed by auto-scaling. Mean changes in sensory perception was assessed by a repeated-measures t-test, performed in Minitab 19. Replicates per analysis are presented in supplemental table S1.

3. Results

Profile analyses (vegetative, yield, and quality)

A range of growth, yield, and quality measures were taken per plant (27 measures total) to assess the impact of the BC treatment on strawberry plants (Table S4). Principal component analysis (Fig. 1A) and PLS-DA (Fig. 1B), identified clearly distinct 95% confidence regions in the trait profiles between BC and control treated plants, which is consistent with the clustering of sample profiles by treatment within the dendrogram (Fig. 1C) and heatmap (Fig. S1). Heatmap analysis (Fig. S1 and Table S5) identified two clusters within the 27 plant measures (13 vegetative, 7 yield, and 8 quality), one cluster with minimal difference between treatments (6 vegetative, 5

Table 2 - Sensory perception scoring matrix

	Sensory perception								
	Poor				Acceptable				Optimal
Aroma - Desirability	1	2	3	4	5	6	7	8	9
Taste - Desirability	1	2	3	4	5	6	7	8	9
	Low				Moderate				High
Mouthfeel - Firmness	1	2	3	4	5	6	7	8	9
Mouthfeel - Juiciness	1	2	3	4	5	6	7	8	9
	None				Moderate				Strong
Aroma - Intensity	1	2	3	4	5	6	7	8	9
Taste - Sweet	1	2	3	4	5	6	7	8	9
Taste - Sour/acid	1	2	3	4	5	6	7	8	9
Taste - Intensity	1	2	3	4	5	6	7	8	9

yield, and 4 quality), and one cluster of measures with large difference between treatments (7 vegetative, 2 yield, and 4 quality). The cluster associated with large differences was comprised of the measures: leaf count (number), leaf fresh weight (g f.w.), leaf dry weight (g d.w.), above ground (non-leaf) fresh weight (g f.w.), above ground (non-leaf) dry weight (g d.w.), canopy area (cm^2), and leaf colour-a (V2, V3, V4, V6, V7, V9, and V11, respectively), total fruit harvested (g) and fruit harvested (number) (Y5 and Y6, respectively), and fruit GAE per fruit dry weight ($g \cdot g^{-1}$ d.w.), Brix %, fruit pH, and fruit water % (Q1, Q2, Q3, and Q5, respectively).

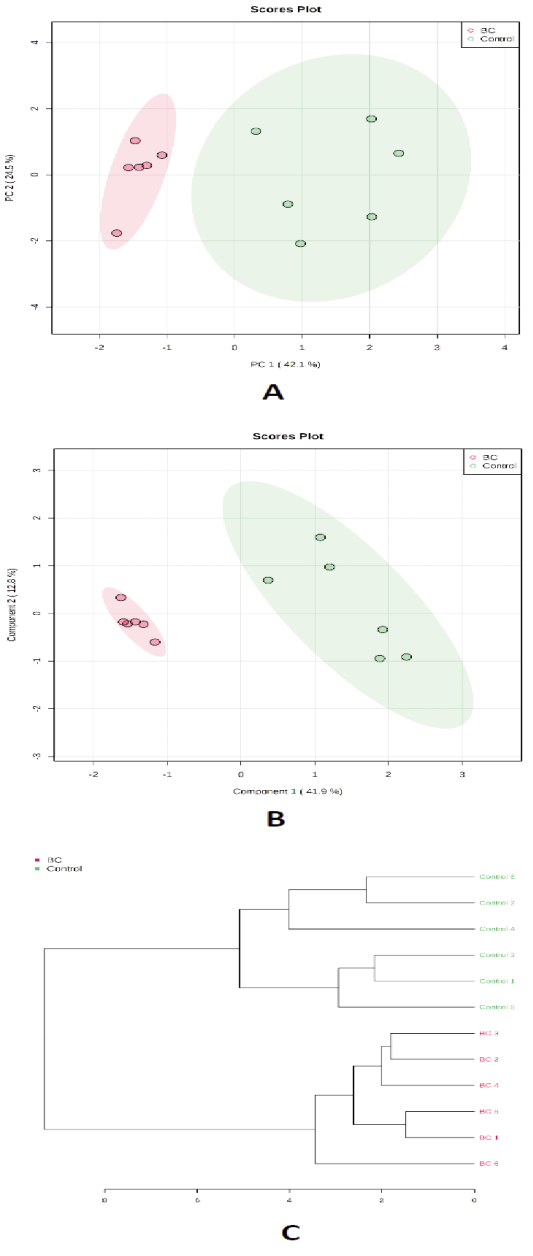

Fig. 1 - Effect of biostimulant complex (BC) on strawberry (*Fragaria x ananassa* 'Albion') trait profiles. (A) Principal component analysis (PCA), and (B) partial least squares-discriminant analysis (PLS-DA), with shading indicating 95% confidence regions. C) Dendrogram indicating hierarchical clustering (Ward clustering algorithm) based on Euclidean distance of plants based on measured traits. Trait values measured from 6 biological replicates.

Vegetative analyses

Treatment with BC had significant impacts on several vegetative measures (Table S4). The greatest effect was seen for leaf measures including: leaf number ($p < 0.001$) which increased by 107.4% (over two-fold) from 15.67 in control to 32.5 in treatment (Fig. 2A), leaf dry weight ($p = 0.002$) which increased by 66.7% from 7.4 g d.w. in control to 12.4 g d.w. in treatment (Fig. 2B), and canopy area ($p = 0.001$) which increased by 51.8% from 401.4 cm^2 in control to 609.3 cm^2 for treatment (Fig. 2C). Whilst vegetative measures tended to increase, no significant change ($p = 0.205$) was identified for leaf water content (Table S4). Furthermore, the BC treatment increased ($p = 0.001$) non-leaf aerial dry weight by 64% from 5.7 g d.w. for control to 9.4 g d.w. for treatment (Table S4), and with marginal significance ($p = 0.057$) increased crown number by 35% from 3.3 to 4.5 (Fig. 2D), whilst not significantly affecting leaf

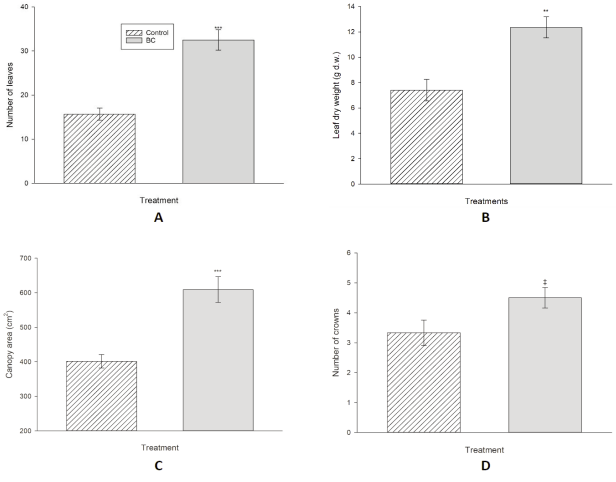

Fig. 2 - Effect of biostimulant complex (BC) treatment on vegetative growth measures of strawberry (*Fragaria x ananassa* 'Albion'). A) Number of leaves, (B) leaf dry weight, (C) canopy area, and (D) number of crowns compared between control and BC treated plants. Data presented mean ± standard deviation of 6 biological replicates. Significant differences are indicated by ‡ for $p < 0.1$; * for $p < 0.05$; ** for $p < 0.01$; *** for $p \leq 0.001$ calculated by Student's t-test.

colour measures *L**, *a**, and *b** (Table S4).

Yield analyses

The biostimulant complex significantly increased the yields of strawberry plants in terms of total weight of fruits per plant (p = 0.038), and number of fruits per plant (p = 0.035), whilst not significantly (p = 0.666) affecting individual fruit weight (Table S4). The average weight of total fruits harvested per plant increased by 50.7% from 128.3 g for control to 193.3 g for treatment (Fig. 3A), while the average number of fruits harvested per plant increased by 56.9% from 12.0 for control to 18.8 for treatment (Fig. 3B). Whilst no changes were observed for individual fruit weight, changes to fruit shape were observed wherein BC treatment significantly (p = 0.013) increased fruit length (Table S4) from 30.33 mm to 35.48 mm (17% increase), whilst fruit width was unchanged (p = 0.446). Harvest timing was normally distributed for control plants (p = 0.268) but not for treatment plants (p = 0.038), however no significant difference between treatments was identified in median fruit harvest timing (p = 0.971, Fig. 3C).

Fruit quality analyses

Chemical analysis of strawberry fruits identified that BC treatment significantly increased fruit water content (p = 0.001) wherein fruit water content from treatment plants was 90.2%, whilst fruits from control plants had 89.06% water content (Fig. 4A). Additionally, the BC treatment resulted in significant changes to fruit image colour profiles, wherein R% increased from 29.66% for control to 39.22% (p = 0.001), G% increased from 0.38% for control to 1.01% (p = 0.001), and O% decreased from 28.90% for control to 20.79% (p = 0.006), whilst Y% (p = 0.371) and A% (p = 0.186) were not significantly impacted (Fig. 4B). The fruit colour measures B, Pi, C, W, and Pu accounted for on average less than 0.01% of pixels within images and so were not explored during analyses. No significant difference was observed for Brix content (p = 0.941) between treatment or control plants (Fig. 4C). The biostimulant complex treatment resulted in a marginally (defined as 0.1 < p < 0.05) significant (p = 0.055) increase to pH of diluted crude fruit extract, from 4.2 to 4.4 (Table S4).

It was identified that the BC treatment had significant affects (p = 0.029) on TP content (Fig. 4D) with a 32% increase from 0.0139 GAE $g \cdot g^{-1}$ d.w. for control to 0.0184 GAE $g\ g^{-1}$ d.w. for treatment, whilst not significantly (p = 0.534) affecting flavonoid content (Fig. 4E). Fruit quality perception was analysed by a blinded sensory perception test on a 9-point scale, which identified that the BC treatment significantly (p = 0.043) increased fruit mouthfeel firmness ('firmness'), from 4.2 for control to 5.5 for treatment, whilst not significantly impacting mouthfeel juiciness ('juiciness'), or fruit taste measures (Table S6).

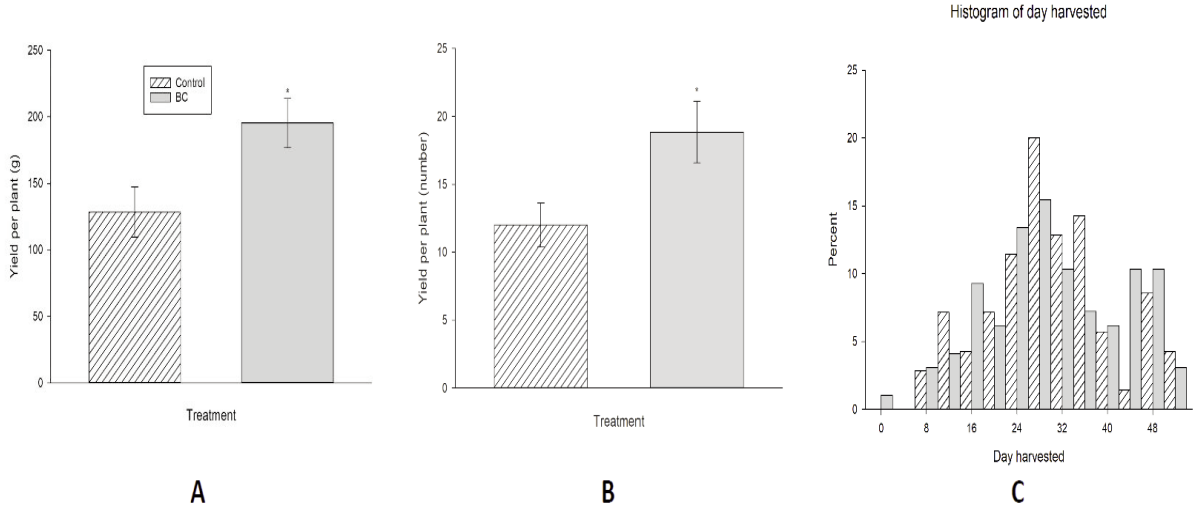

Fig. 3 - Effect of biostimulant complex (BC) treatment on yield and harvest timing of strawberry fruits (*Fragaria x ananassa* 'Albion'). Fruit yield per plant by (A) weight, and (B) count compared between control and BC treated plants. A-B) Data presented as mean ± standard deviation of 6 biological replicates. Significant differences are indicated by * for p < 0.05 calculated by Student's t-test. C) Histogram of harvest timing from 72 fruits from control plants and 97 fruits from BC treated plant.

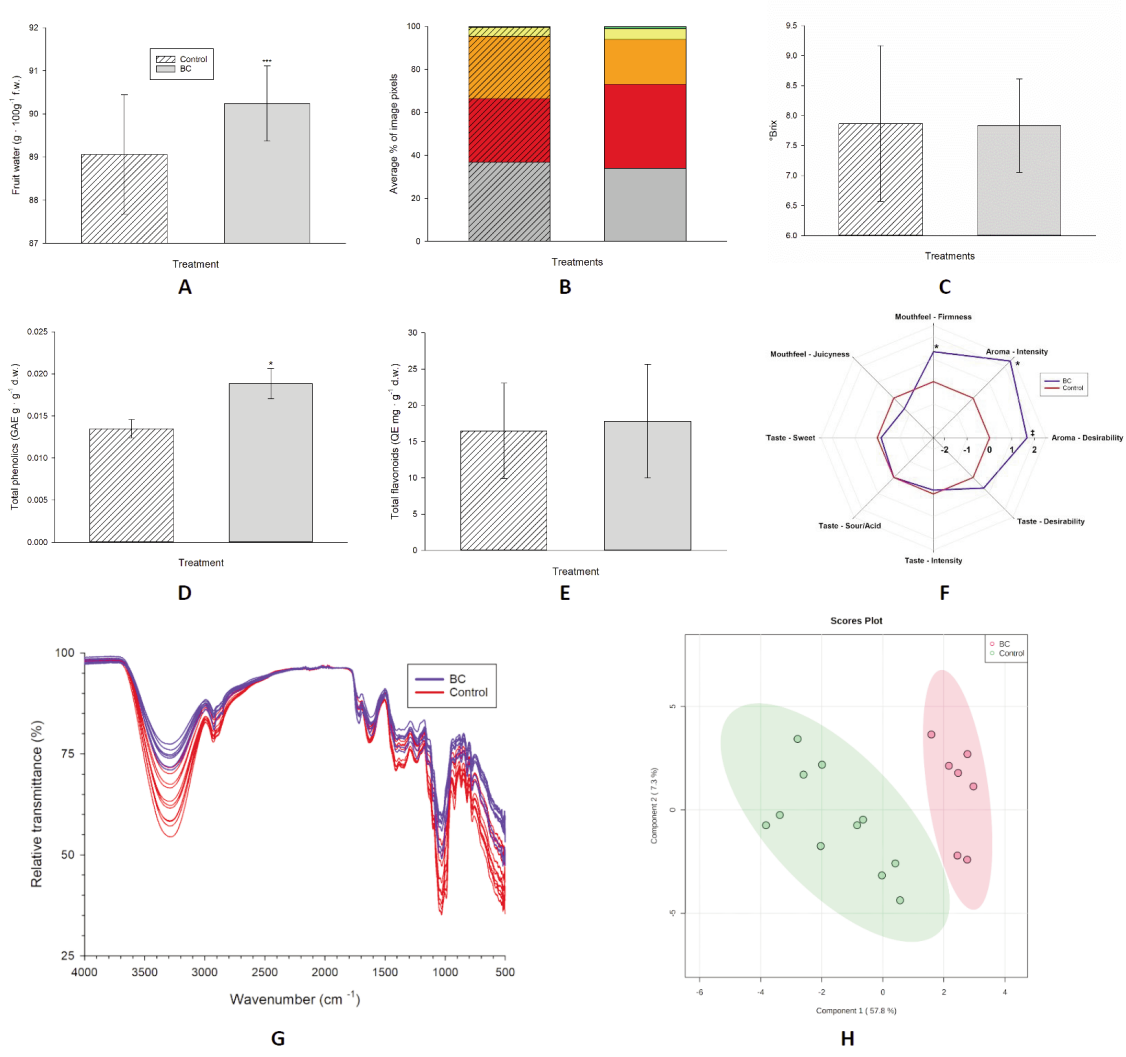

Fig. 4 - Effect of biostimulant complex (BC) treatment on quality and sensory perception of strawberry (*Fragaria* x *ananassa* 'Albion') fruits. A) Fruit water (22 and 27 biological replicates for control and BC, respectively), (B) colour profile (22 and 27 biological replicates for control and BC, respectively with each colour represented as their respective colour), (C) Brix (6 and 13 biological replicates for control and BC, respectively), (D) total phenolics (22 and 27 biological replicates for control and BC, respectively), and (E) total flavonoids (22 and 27 biological replicates for control and BC, respectively) compared between control and BC treated plants. A, C-E) Data represented as mean ± standard deviation. F) Comparison of mean scores (6 participants) for blind sensory perceptions of fruit from control and BC treated plants. A-E) Solid bars represent control and dashed bars represent BC treatment. A, C-F) Significant differences are indicated by ‡ for $p < 0.1$; * for $p < 0.05$; ** for $p < 0.01$; *** for $p \leq 0.001$ calculated by Student's t-test (A-E), and repeated-measures t-test (F). G) Mid-infrared (M-IR) spectra of dehydrated strawberry fruits analysed between 500-4000 cm^{-1} (7 and 11 biological replicates for control and BC, respectively). H) Sparse partial least squares-discriminant analysis (sPLS-DA) of M-IR spectra with shading indicating 95% confidence regions.

Furthermore, the BC treatment significantly (p = 0.040) increased fruit aroma intensity from 3.5 to 5.2, and had a marginally significant (p = 0.067) effect to aroma desirability which increased from 5.0 to 6.7 (Fig. 4F).

Mid-infrared spectrometry analysis between 500-4000 cm^{-1} identified biostimulant induced changes to fruit chemical composition (Fig. 4G). Principle component analysis (PCA: Fig. S2) and PLS-DA (Fig. S3) did not identify significant differences between the profile of spectral bins between treatment groups, while sPLS-DA (Fig. 4H) indicated significant differences in a subset of the wavelength's measured. Comparison of the average spectra of each treatment (Fig. S4), identified a region between 1024-1048 cm^{-1} with the highest fold change (>1.3) between treatments (Fig. S5).

4. Discussion and Conclusions

Crop productivity is an important factor in food production when considering the growing global population and uncertainties associated with climate change (Lobell and Gourdji, 2012). Accordingly, new strategies to increase crop outputs are highly sought after, with a focus on fast acting benefits which do not contribute to environmental degradation. Whilst genetic modification (GM) continues to benefit many crop sectors, the costs, time, and resources required to develop approved GM food crops is a significant hurdle. Accordingly, biostimulants are becoming increasingly popular additives during plant growth due to their benefits to crop productivity, natural origin, cost, and ease of use (Parađiković *et al.*, 2019). Herein a naturally derived biostimulant complex comprising molasses, *Aloe vera* extract, and fish-hydrolysate exemplifies these beneficial effects by increasing the growth and yield of strawberry.

Application of the BC was shown to increase vegetative biomass and canopy area measures (Fig. 2A-C), potentially associated with the provision of zeatin (cytokinin) - the only phytohormone detected in both the BC concentrate and its associated reservoir solution (Table S2) - which has been shown to increase shoot and root growth when applied exogenously to strawberry (Debnath, 2006). Additionally, the BC treatment resulted in a marginally significant (p = 0.057) increase to crown number (Fig. 2D). The crown is the central node of the strawberry plant from which roots, leaves, inflorescence, and additional crowns form (Savini *et al.*, 2005; Poling, 2012). As crowns are the base of future inflorescence formation, their number correlates strongly with fruit yield (Strik and Proctor, 1988; Kadir *et al.*, 2006) and is therefore an important factor for strawberry cultivation. Additionally, application of the BC was observed to increase both yield-weight (Fig. 3A, p = 0.038) and yield-number (Fig. 3B, p = 0.035) per plant - potentially associated with the increased crown number - which are crucial measures of profitability for farmers. Accordingly, the increases in yield outputs reported herein (Fig. 3A and 3B) support the utilisation of these biostimulants by the strawberry industry and thereby presents as a low-cost, effective, and easily integrated farming strategy to improve growth and yield.

Furthermore, changes to strawberry shape - increased length (Fig. S6A, p = 0.013) but not weight (Fig. S6B, p = 0.666) or width (Fig. S6C, p = 0.446) - was observed from application of the BC. Changes to fruit size and shape can be impactful to farmer sales due to the compliance standards imposed by supermarkets. For strawberry fruits, compliance is generally determined according to diameter (USDA, 2006; Woolworths Supermarkets Ltd, 2010), which is also the highest correlating size measurement (R^2 = 0.93) with consumer preference, however, length is the second highest correlation (R^2 = 0.77) (Lewers *et al.*, 2020), suggesting that longer fruits of unchanged width may be considered preferable by consumers. Accordingly, application of this biostimulant complex to strawberries during growth can benefit farmers by improving yield and improve customer perceptions of quality through altered fruit size.

Whilst the aforementioned changes to fruit size are likely to be impactful to consumer perception of fruit quality, organoleptic properties and colour features are also highly correlative with strawberry quality perception (Lewers *et al.*, 2020). Organoleptic measures include taste, texture, mouthfeel, and aroma, which are conferred to the fruit through its chemical composition (Saliba-Colombani *et al.*, 2001). Common measures associated with taste include soluble solids content (SSC), titratable acidity (Wozniak *et al.*, 1996), and pH (Gunness *et al.*, 2009). Herein no significant change was detected for brix (p = 0.941), a measure of SSC (Saranwong *et al.*, 2003), which is consistent with the results from the sensory assessment wherein no significant change (p = 0.822) was observed in the correlated measure, sweetness (Jouquand *et al.*, 2008). Similarly, sour perception may have been expected to change with pH (Jouquand *et al.*, 2008), and whilst a marginally significant change was observed for fruit pH (p = 0.055), no significant change was reported from panellists for the sensory measure sour (p = 1.000). This may be explained by the apparently small change of 0.11 pH of diluted extract, which is consistent with the findings of Harker *et al.* (2002) wherein a minimum shift of 0.14 pH of apple extract was required for participants to perceive a change in apple acidity. These results suggest that utilisation of the BC during strawberry cultivation may increase yield without compromising quality. Noting that a growing point of consumer dissatisfaction is the reduction in food flavour and aroma due to the prioritisation of more profitable crop attributes such as yield and visual aesthetic (Klee, 2010; Tieman *et al.*, 2017), these results support the utilisation of biostimulants as being advantageous to both farmers and consumers.

Fruit mouthfeel and texture are associated with cell wall composition (Caner *et al.*, 2008) and thickness (Szczesniak and Smith, 1969), water content (Cordenunsi *et al.*, 2002), and pH (Plotto *et al.*, 2010). Additionally, Salentijn *et al.* (2003) and Wang *et al.* (2021) have shown that increased expression of genes associated with lignin production is associated with increased firmness of strawberry fruit. Accordingly, the significant increase in mouthfeel-firmness (p = 0.043) may relate to the presence of caffeic acid in the BC (Table S3), which may internally translocate via the phloem and xylem (Zhang and Hamauzu, 2004; Ishimaru *et al.*, 2011) and has been shown to increase lignin production in soybean (Bubna *et al.*, 2011). Richter (1978) identified that plant cell turgidity is highly sensitive to changes in relative water content (RWC), with flaccidity (loss of turgidity) to full turgor occurring over the narrow range of 5% RWC. Accordingly, as fruit firmness is impacted by turgidity (Szczesniak and Smith, 1969; Raharjo *et al.*, 1998), the 1% increase in fruit water content (p = 0.001) identified from the BC treatment may explain the observed increase in the sensory measure for firmness. Whilst this apparently minor change in fruit water content (Fig. 4A) may have impacted perceived firmness (Fig. 4F), it is however not surprising that this small change in fruit water volume (150 µL, based on 1% of 10.5 g average fruit fresh weight) was below sensory perception thresholds to impact perceived juiciness, as juiciness is generally considered as the amount of liquid released during chewing (Roger Harker *et al.*, 2003; Harker *et al.*, 2006).

Aroma - also referred to as odour - is the detection and recognition of compounds within the olfactory system and is conferred by the presence of volatile compounds (El Hadi *et al.*, 2013). In strawberry, aroma is predominantly attributed to esters, furanones, terpenes, and sulfur compounds (Yan *et al.*, 2018). As with fruit flavour, aroma is often seen by consumers as a sacrifice for higher yields (Klee, 2010; Tieman *et al.*, 2017), which necessitates the need for methods to improve aroma, or improve yields without compromising this measure. Herein a significant difference (p = 0.040) was observed for aroma intensity and a marginally significant (p = 0.067) difference was observed for aroma desirability (Fig. 4F), suggesting that the BC treatment may have altered the volatile contents or profiles of the fruits. The M-IR analysis presented in figure 4G revealed a narrow region between 1024–1048 cm^{-1} with a high fold change, which has been associated with chemicals in the classes of phenolic alkyl-aryl ethers, aryl phenolic ester tannins (Abbas *et al.*, 2017), pyranose rings (saccharides), alkyl amines, and alcohols (Lingegowda *et al.*, 2012). Furthermore, of these classes of compounds, esters are one of the most abundant volatiles in strawberries (Yan *et al.*, 2018) and have been shown to correlate strongly with strawberry fruit liking (Fan *et al.*, 2021). Whilst the scoring of odour desirability alone herein was only marginally significant, this association with overall fruit liking combined with the other changes reported herein, is likely to contribute to an overall improvement to fruit quality perception from the BC treatment. Accordingly, BC treatment may have resulted in changes to ester levels to enhance the sensory aroma properties of strawberry fruits, which is also reflected by M-IR profile changes over a narrow region. These outcomes address consumer concerns for losses in aroma associated with prioritisation of more profitable traits, by demonstrating that BC treatment increases both yield and aroma.

Finally, fruit appearance, which includes colour (Crisosto *et al.*, 2003) and damage (Jaeger *et al.*, 2018), is a major impactor to consumer perception of quality and purchasing decision, as it is the first impression of a fruit. Biostimulant complex treatment resulted in significant increases to the colour measures for red (p = 0.001) and green (p = 0.001) and reductions in orange (p = 0.006). Strawberry colour is conferred by the presence (amount and types) of anthocyanins, which have a strong pH-colour relationship (Holcroft and Kader, 1999). The predominant anthocyanins in strawberry are pelargonidins and cyanidins (Andersen *et al.*, 2004) which appear red at low pH and with increasing pH change to colourless, yellow, or blue forms which affects the overall appearance of the fruit (Holcroft and Kader, 1999). Whilst a marginally significant (p = 0.055) increase of 0.11 pH in diluted fruit extract was associated with the BC treatment, this degree of change is small relative to the change observed in Wang *et al.* (2015) wherein pH shifts of 1.0 were associated with noteworthy changes to colour. Accordingly, pH is likely not the driver of the observed changes in fruit colour, which may instead be attributed to changes in the concentrations or ratios of anthocyanins present (Yoshida *et al.*, 2002). Nevertheless, strawberry colour is a driver of consumer preference, as evidence by Wang *et al.* (2017) wherein an 'ideal red' colour was the preference for

fresh strawberry fruit, and by Wendin *et al.* (2019) which showed that red colour intensity had a significant positive impact to consumer preference for woodland strawberries. These studies suggest that the increased red from BC treatment reported herein (Fig. 4B) for common garden strawberries may also be associated with increased consumer preference.

Unlike organoleptic measures and colour features which are directly detectible by consumers and therefore impactful to quality perception and preference, other properties such as nutritional and functional food value should also be considered as targets for improvement during production and cultivation as their increased presence may benefit consumer health (Selby-Pham *et al.*, 2017; Topolska *et al.*, 2021). Due to the presence of many beneficial polyphenols and vitamins, strawberries are considered to be a functional food which can reduce hypertension, postprandial oxidative stress, inflammation, and hyperglycaemia when consumed (Giampieri *et al.*, 2015). Furthermore, agricultural practises such as fertiliser form (Tomic *et al.*, 2016), cultivation system (D'evoli *et al.*, 2010), and beneficial microbes (Rahman *et al.*, 2019) have been shown to impact phytochemical profiles and antioxidant activities in strawberries. Accordingly, the results presented herein are similar to these observations, wherein altered cultivation conditions through application of the BC was shown to impact phytochemical concentrations which are associated with functional activity when consumed.

Herein two methods were utilised to measure functional compounds in strawberry, and whilst the aluminium chloride method has relatively good specificity for flavonoid quantification (Mabry *et al.*, 1970), the F-C method is a non-specific method, which quantifies total reducing capacities (antioxidant activity) rather than specific classes of compounds (Magalhães *et al.*, 2008). Accordingly, the 32% increase (p = 0.029) in TP and unchanged (p = 0.534) flavonoid contents (Fig. 4D and 4E, respectively) reported herein indicates that the biostimulant treatment increased the antioxidant activity of the fruits in the non-flavonoid portion of the phytochemical profile. Aaby *et al.* (2007) identified that the largest contributors to strawberry antioxidant activity were ascorbic acid, and the polyphenolics ellagitannins, and anthocyanins, which accounted for 24%, 19%, and 13% of strawberry antioxidant capacities, respectively. Anthocyanins are also the class of compounds conferring the majority of strawberry colour (Yoshida *et al.*, 2002), which also changed in response to the biostimulant application (Fig. 4B), discussed above. As noted, changes in anthocyanin concentrations or ratios may explain the changes in colour observed for biostimulant treated fruits, and changes to anthocyanins may also affect antioxidant activities of the fruit extracts (Cerezo *et al.*, 2010). The distinguishing feature of anthocyanins, is their multiple aromatic rings with hydroxyl groups (phenolic) structure, derived from the flavylium ion (Khoo *et al.*, 2017). Whilst the carbon bonds of these aromatic rings (C=C) and the hydroxyl groups (OH) are associated with IR absorption at 1654 cm^{-1} and 677 cm^{-1}, and 3385 cm^{-1}, respectively, the C-O bond connecting the hydroxyl to the aromatic ring is associated with wavenumber 1029 cm^{-1} (Wahyuningsih *et al.*, 2017), which is contained within the range of wavenumbers (1024-1048 cm^{-1}) identified herein as having increased from the BC treatment (Fig. 4H and S5). Accordingly, it appears that the BC treatment induced changes in the strawberry anthocyanin content or profile, which would be consistent with the changes observed for TP (Fig. 4D), colour (Fig. 4B), and M-IR spectra (Fig. 4G). Furthermore, the changes in TP (antioxidant activity) correspond with improved functional food potential of these fruits (Giampieri *et al.*, 2015), which may impart greater health benefits than control strawberries when consumed. Implementation of this complex is therefore a promising improvement to strawberry cultivation practises and may be an additional tool available to farmers to improve yields and quality of produce for consumers.

Biostimulants are an exciting development in agriculture which have the potential to improve crop yields and quality, whilst not requiring significant time and money to substantially alter crop outputs, by contrast to alternative strategies such as genetic modification and selective breeding. However, species-specific efficacies of popular biostimulants even when applied to commonly grown food crops are often not well understood. Accordingly, this project characterised the impacts of a complex containing the biostimulants molasses, *Aloe vera* extract, and fish-hydrolysate when applied to strawberry in a hydroponic, environmental-controlled growth system. The results demonstrated that application of the complex increased crop yield, vegetive growth, and fruit quality measures including aroma and functional food value. Accordingly, utilisation of biostimulants within farming practises

has demonstrated potential to increase crop outputs for farmers whilst enhancing the quality of foods for consumers.

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Supplementary materials

A biostimulant complex comprising molasses, *Aloe vera* extract, and fish-hydrolysate enhances yield, aroma, and functional food value of strawberry fruit

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Abbreviations: A = achromatic; ATC = automatic temperature compensation; B = blue; BC = Biostimulant complex; C = cyan; EC = electrical conductivity; F-C = Folin-Ciocalteu; FTIR = Fourier transformed infrared; G = green; GAE = gallic acid equivalent; GLM = general linear model; GM = genetic modification; M-IR = mid-infrared; O = orange; PCA = principle component analysis; Pi = pink; PLS-DA = partial least squares-discriminant analysis; Pu = purple; QE = quercetin equivalent; R = red; RWC = relative water content; sPLS-DA = sparse partial least squares-discriminant analysis; SSC = soluble solids content; TP = total phenolics; UATR = universal attenuated total reflectance; W = white; Y = yellow.

Abstract: Strawberry is a popular functional food due to the presence of antioxidant and anti-inflammatory phytochemicals. Enhancing this functional food value is an opportunity to improve consumer health, but strategies to do so cannot compromise yield or organoleptic properties, which are highest priorities for farmers and consumer, respectively. One promising strategy is the supplementation of fertiliser regimens with biostimulants, which are non-nutritive substances associated with species-specific improvements to crop growth, yield, and quality. Accordingly, the impacts of a biostimulant complex (BC) containing molasses, *Aloe vera* extract, and fish-hydrolysate is characterised herein for its potential to impact strawberry growth, yield, quality, and functional food value. Results indicated that BC treatment significantly increased ($p < 0.05$) plant biomass and canopy area (growth), total fruit count and weight per plant (yield), fruit aroma and colour (quality), and antioxidant potential (functional food value). The results presented highlight the potential utility of biostimulants to the strawberry sphere, providing a strategy to enhance the fruit to the benefit of both farmers and consumers.

Table S1 - Nutrient profile of biostimulant comples (BC)

Nutrient	Content (mg· L^{-1})
Nitrogen[A]	2600
Phosphorus[B]	454.25
Potassium[B]	10230.5
Sulfur[A]	4400
Calcium[B]	592.5
Magnesium[B]	2133
Silica (SiO_2) [B]	211.3
Boron[B]	<50
Iron[B]	<50
Zinc[B]	<50
Manganese[B]	<50

A = Quantified via LECO TruMac CNS Analyser as per Rayment, G.E., Lyons, D.J., 2011. Soil chemical methods: Australasia. CSIRO publishing.
B = ICP/AES by ACS Laboratories, Victoria, Australia.

Table S2 - Phytohormone profile of biostimulant complex (BC) and reservoir solutions

Phytohormone	BC ($ng \cdot ml^{-1}$)	BC reservoir ($ng \cdot ml^{-1}$)	Control reservoir ($ng \cdot ml^{-1}$)
Gibberellin A3	0.000	0.000	0.000
Gibberellin A4	0.000	0.000	0.000
Indole-3-acetic acid (IAA)	0.000	0.000	0.000
Methyl-IAA	0.000	0.000	0.000
Indole-3-carboxylic acid (ICA)	0.000	0.000	0.000
Zeatin (cytokinin)	70.362	5.251	0.000
Abscisic acid (ABA)	121.043	0.000	0.000
Jasmonic acid (JA)	15.824	0.000	0.000
Methyl-JA	0.000	0.000	0.000
JA-Isoleucine	4.077	0.000	0.000
12-Oxo-phytodienoic acid (OPDA)	42.937	0.000	0.000
Salicylic acid	822.589	0.000	0.000
Cinnamic acid	123.408	0.000	0.000
Brassinolide	0.000	0.000	0.000

Phytohormone quntification performed as per: Suwanchaikasem P., Idnurm A., Selby-Pham J., Walker R., Boughton B.A., 2022 - *Root-TRAPR: a modular plant growth device to visualize root development and monitor growth parameters, as applied to an elicitor response of* Cannabis sativa. - Plant methods, 18(1): 1-20.

Table S3 a - Metabolic profile of biostimulant complex (BC)

Name	Formula	Molecular weight	RT [min]	Peak area
(-)-Spiculisporic acid	C17 H28 O6	328.189	31.781	187.48
(+)-streptol	C7 H12 O5	1.760.684	3.246	2.64
(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	C18 H34 O5	33.024.104	29.362	32.94
(1S,3R,4R,5R)-1,3,4-trihydroxy-5-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]oxy}cyclohexane-1-carboxylic acid	C17 H20 O9	36.811.126	24.763	45.92
(2R)-2-Hydroxy-3-[(hydroxy{[(1S,2R,3R,4S,5S,6R)-2,3,4,5,6-pentahydroxycyclohexyl]oxy}phosphoryl)oxy]propyl palmitate	C25 H49 O12 P	5.722.965	32.936	38.69
(2R,3S)-3-isopropylmalic acid	C7 H12 O5	17.606.842	3.092	1.34
(3R,5R)-1,3,5-Trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid	C17 H20 O9	36.811.125	19.92	54.62
(3R,5R)-1,3,5-Trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid	C17 H20 O9	36.811.129	24.335	36.45
(3S,4S)-3-Hydroxy-1,3,4-tetradecanetricarboxylic acid	C17 H30 O7	34.619.955	31.211	28.56
(DL)-3-O-Methyldopa	C10 H13 N O4	21.108.455	17.674	9.67
[7-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-6-methoxy-1,2,3,4-tetrahydro-2-naphthalenyl]methyl pentofuranoside	C25 H32 O10	49.220.024	26.447	1.22
1-(2-Deoxypentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione	C10 H14 N2 O5	24.209.055	2.376	0.73
1-(3-Acetyl-2,4,6-trihydroxyphenyl)-1,5-anhydrohexitol	C14 H18 O9	33.009.558	10.077	42.08
1-(6-hydroxy-3-pyridyl)-4-(methylamino)butan-1-one	C10 H14 N2 O3	21.010.054	24.059	0.34
1,5-Anhydro-1-[5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-4-oxo-4H-chromen-8-yl]hexitol	C23 H24 O11	47.613.266	26.635	14.63
1-oleoylglycerone 3-phosphate	C21 H39 O7 P	43.424.396	33.182	56.83
1-Palmitoyl lysophosphatidic acid	C19 H39 O7 P	41.024.397	33.428	39.08
2-(2,4-Dihydroxyphenyl)-5,7-dihydroxy-6-(3-hydroxy-3-methylbutyl)-4H-chromen-4-one	C20 H20 O7	37.212.146	28.055	7.63
2-(Acetylamino)hexanoic acid	C8 H15 N O3	17.310.509	16.179	3.41
2-(Acetylamino)hexanoic acid	C8 H15 N O3	17.310.508	14.583	3.37
2,3-Dihydro-1-benzofuran-2-carboxylic acid	C9 H8 O3	16.404.707	23.039	67.63
2,5-Dihydroxybenzaldehyde	C7 H6 O3	13.803.173	7.814	40.62
2-[7,8-Dihydroxy-6-(hydroxymethyl)-2-methylhexahydro-4aH-pyrano[2,3-b][1,4]dioxin-2-yl]-5-methylcyclohexanone	C16 H26 O7	3.301.682	31.454	15.07
2-Hydroxy-3-[3-(3-methyl-2-buten-1-yl)-4-(sulfooxy)phenyl]propanoic acid	C14 H18 O7 S	33.007.778	25.808	0.45
2-Hydroxycaproic acid	C6 H12 O3	13.207.844	8.296	48.17
2-Hydroxycaproic acid	C6 H12 O3	13.207.842	9.409	38.20
2-hydroxysebacic acid	C10 H18 O5	21.811.568	24.571	9.83
2-Isopropylmalic acid	C7 H12 O5	17.606.851	7.107	36.51
2-Methyl-3-hydroxybutyric acid	C5 H10 O3	1.180.628	3.327	8.95
2-O-caffeoylglucaric acid	C15 H16 O11	3.720.696	24.385	28.00
3-(4-Hydroxy-3,5-dimethoxyphenyl)-2-oxiranecarboxylic acid	C11 H12 O6	24.006.387	3.167	0.10
3-(6,7-Dimethoxy-1,3-benzodioxol-5-yl)-2-oxiranecarbaldehyde	C12 H12 O6	25.206.357	25.767	0.14
3-(6,7-Dimethoxy-1,3-benzodioxol-5-yl)-2-propen-1-ol	C12 H14 O5	2.380.843	24.242	12.62

Secondary metabolite profiling performed using Reverse Phase (RP) HPLC-HRFTMS by The Australian Wine Research Institute (AWRI).

Table S3 b - Metabolic profile of biostimulant complex (BC)

Name	Formula	Molecular weight	RT [min]	Peak area
3,5-Dihydroxy-2-[3-(3-hydroxy-4-methoxyphenyl)propanoyl]phenyl hexopyranosiduronic acid	C22 H24 O12	48.012.732	26.843	13.86
3-[3-(beta-D-Glucopyranosyloxy)-2-hydroxyphenyl]propanoic acid	C15 H20 O9	3.441.112	14.876	13.61
3-Methoxy-4-hydroxyphenylglycol glucuronide	C15 H20 O10	36.010.629	7.82	31.24
3-O-methylgallic acid	C8 H8 O5	18.403.706	12.221	120.37
3-Phenyllactic acid	C9 H10 O3	1.660.628	14.906	27.57
4-(2-Carboxyethyl)-2-methoxyphenyl beta-D-glucopyranosiduronic acid	C16 H20 O10	37.210.607	22.831	85.21
4-(2-Carboxyethyl)-2-methoxyphenyl beta-D-glucopyranosiduronic acid	C16 H20 O10	37.210.594	17.893	10.81
4-Hydroxybenzaldehyde	C7 H6 O2	1.220.367	11.398	38.26
4-Oxoproline	C5 H7 N O3	1.290.424	1.261	68.37
4-Oxoproline	C5 H7 N O3	12.904.227	1.478	0.18
5-(3',5'-Dihydroxyphenyl)-gamma-valerolactone 3-O-glucuronide	C17 H20 O10	38.410.614	24.376	24.80
6-O-sinapoyl-D-glucono-1,5-lactone	C17 H20 O10	38.410.631	15.108	7.78
Arachidonic acid	C20 H32 O2	30.424.055	33.885	0.03
asp-leu	C10 H18 N2 O5	24.612.169	4.442	4.61
Azelaic acid	C9 H16 O4	18.810.508	25.675	16.38
Caffeic acid	C9 H8 O4	18.004.246	17.113	14.00
Catalpol	C15 H22 O10	36.212.169	12.848	153.83
Chelidonic acid	C7 H4 O6	18.400.088	1.34	4.34
Chlorogenic acid	C16 H18 O9	35.409.543	20.664	130.00
Choline O-Sulfate	C5 H13 N O4 S	18.305.659	0.934	2.18
Citraconic acid	C5 H6 O4	13.002.652	1.151	49.63
Citric acid	C6 H8 O7	19.202.657	1.151	2023.75
Corchorifatty acid F	C18 H32 O5	32.822.539	28.816	12.55
D-(-)-Fructose	C6 H12 O6	18.006.339	1.369	60.40
D-(-)-Fructose	C6 H12 O6	12.004.293	1.128	16.65
D-(-)-Quinic acid	C7 H12 O6	19.206.351	0.945	18.95
DL-4-Hydroxyphenyllactic acid	C9 H10 O4	18.205.822	7.4	11.00
DL-Malic acid	C4 H6 O5	13.402.141	1.016	211.49
D-Xylonic acid	C5 H10 O6	16.604.796	0.929	119.88
D-α-Hydroxyglutaric acid	C5 H8 O5	14.803.703	1.227	54.09
D-α-Hydroxyglutaric acid	C5 H8 O5	14.803.673	1.399	24.65
Eicosapentaenoic acid	C20 H30 O2	30.222.489	33.652	0.01
Fertaric acid	C14 H14 O9	3.260.644	25.81	13.23
Flazin	C17 H12 N2 O4	30.808.017	28.778	10.77
Gallic acid	C7 H6 O5	17.002.136	3.018	806.09
Gentisic acid	C7 H6 O4	15.402.653	5.435	1.52

Secondary metabolite profiling performed using Reverse Phase (RP) HPLC-HRFTMS by The Australian Wine Research Institute (AWRI).

Table S3 c - Metabolic profile of biostimulant complex (BC)

Name	Formula	Molecular Weight	RT [min]	Peak area
Î²-D-Glucopyranuronic acid, 1-(3-methylbutanoate)	C11 H18 O8	27.810.045	6.057	18.49
Imidazoleacetic acid riboside	C10 H14 N2 O6	25.808.548	2.102	5.37
Leu-Val	C11 H22 N2 O3	23.016.357	10.268	13.06
Leu-Val	C11 H22 N2 O3	2.301.634	7.103	6.52
L-Phenylalanine	C9 H11 N O2	16.507.908	2.921	2.52
Malonic acid	C3 H4 O4	10.401.092	1.057	16.78
Malonic acid	C3 H4 O4	1.040.109	1.107	5.35
Methylsuccinic acid	C5 H8 O4	13.204.228	3.259	17.43
MX5800000	C8 H10 O4	17.005.807	4.75	1.61
Myristylsulfate	C14 H30 O4 S	29.418.675	31.153	427.93
N-(3,4-Dihydroxyphenyl)glutamine	C11 H14 N2 O5	25.409.043	25.249	12.57
N-[(2S)-2-Hydroxypropanoyl]-L-tryptophan	C14 H16 N2 O4	2.761.112	22.946	19.67
N-Acetyl-L-methionine	C7 H13 N O3 S	19.106.168	7.388	0.05
N-Acetyl-L-phenylalanine	C11 H13 N O3	20.708.982	21.533	2.78
Neochlorogenic acid	C16 H18 O9	3.540.955	10.497	118.55
Pantothenic acid	C9 H17 N O5	21.911.094	4.822	42.68
Phaseolic acid	C13 H12 O8	29.605.366	24.186	19.41
Pimelic acid	C7 H12 O4	16.007.348	5.721	2.86
Pimelic acid	C7 H12 O4	16.007.346	5.256	2.37
porphobilinogen	C10 H14 N2 O4	22.609.631	7.798	8.79
Quercetin	C15 H10 O7	30.204.315	28.599	0.08
Questiomycin A	C12 H8 N2 O2	21.205.878	23.037	3.53
Saccharin	C7 H5 N O3 S	18.299.877	3.44	12.27
Sakuranin	C22 H24 O10	44.813.762	28.211	6.45
Salicylic acid	C7 H6 O3	13.803.172	21.923	31.37
Seryltyrosine	C12 H16 N2 O5	26.810.603	26.872	13.50
shanzhiside	C16 H24 O11	39.213.231	17.69	79.16
Suberic acid	C8 H14 O4	17.408.925	2.973	1.18
Sucrose	C12 H22 O11	34.211.672	0.949	25.06
Swertiajaponin	C22 H22 O11	46.211.704	25.624	31.75
Trifluoroacetic acid	C2 H F3 O2	11.399.281	1.096	3.80
Valylvaline	C10 H20 N2 O3	21.614.764	2.764	1.75
Vanillin	C8 H8 O3	15.204.728	13.382	18.82
Volkenin	C12 H17 N O7	28.710.087	23.619	1.61

Secondary metabolite profiling performed using Reverse Phase (RP) HPLC-HRFTMS by The Australian Wine Research Institute (AWRI).

Table 4 - Impact of the biostimulant complex (BC) on strawberry growth, yield, and quality

Measure	Average value		P
	Control	BC	
Total fruit harvested (number · plant^{-1})	12.00 (n = 6)	18.83 (n = 6)	0.035
Total fruit harvested (g · plant^{-1})	128.3 (n = 6)	193.3 (n = 6)	0.038
Total unripe fruit (number · plant)	10.33 (n = 6)	11.17 (n = 6)	0.814
Total unripe fruit (g · plant^{-1})	35.18 (n = 6)	27.53 (n = 6)	0.600
Number of crowns	3.333 (n = 6)	4.500 (n = 6)	0.057
Number of leaves	15.67 (n = 6)	32.50 (n = 6)	< 0.001
Leaf fresh weight (g f.w.)	28.49 (n = 6)	50.46 (n = 6)	< 0.001
Leaf dry weight (g d.w.)	7.408 (n = 6)	12.350 (n = 6)	0.002
Leaf water %	74.210 (n = 6)	75.584 (n = 6)	0.205
Aerial (non-leaf) fresh weight (g f.w.)	29.08 (n = 6)	50.46 (n = 6)	< 0.001
Aerial (non-leaf) dry weight (g d.w.)	5.717 (n = 6)	9.383 (n = 6)	0.001
Canopy area (cm^2)	401.4 (n = 6)	609.3 (n = 6)	0.001
Leaf colour (*L**)	24.977 (n = 6)	25.370 (n = 6)	0.390
Leaf colour (*a**)	-4.978 (n = 6)	-4.908 (n = 6)	0.829
Leaf colour (*b**)	5.362 (n = 6)	5.275 (n = 6)	0.812
Brix of crude extract (°Bx)	7.867 (n = 6)	7.831 (n = 13)	0.941
pH of crude extraxt (1/1000 dilution in water)	4.2383 (n = 6)	4.3531 (n = 13)	0.055
Fruit length (mm)	30.33 (n = 15)	35.48 (n = 21)	0.013
Fruit width (mm)	25.27 (n = 10)	26.50 (n = 12)	0.446
Fruit fresh weight (g f.w.)	10.691 (n = 72)	10.354 (n = 112)	0.666
Fruit quality compliance (% fruit count with FW > 10 g)	43.1 (n = 72)	44.6 (n = 112)	0.832
Fruit dry weight (g d.w.)	1.1193 (n = 22)	0.9317 (n = 27)	0.069
Fruit water %	89.061 (n = 22)	90.240 (n = 27)	0.001
Fruit total solids %	10.939 (n = 22)	9.760 (n = 27)	0.001
Fruit extract (g·g^{-1} d.w.)	7.126 (n = 22)	7.887 (n = 27)	0.068
Total polyphenolics (GAE g · g^{-1}d.w.)	0.01391 (n = 22)	0.01835 (n = 27)	0.029
Total flavonoids (QE mg · g^{-1}d.w.)	16.48 (n = 22)	17.80 (n = 27)	0.534
Harvest timing (days)	30.16 (n = 72)	30.17 (n = 97)	0.992
Fruit image colour A%	36.77 (n = 22)	33.92 (n = 27)	0.186
Fruit image colour O%	28.90 (n = 22)	20.79 (n = 27)	0.006
Fruit image colour G%	0.3831 (n = 22)	1.008 (n = 27)	0.001
Fruit image colour Y%	4.282 (n = 22)	5.061 (n = 27)	0.371
Fruit image colour R%	29.66 (n = 22)	39.22 (n = 27)	0.001

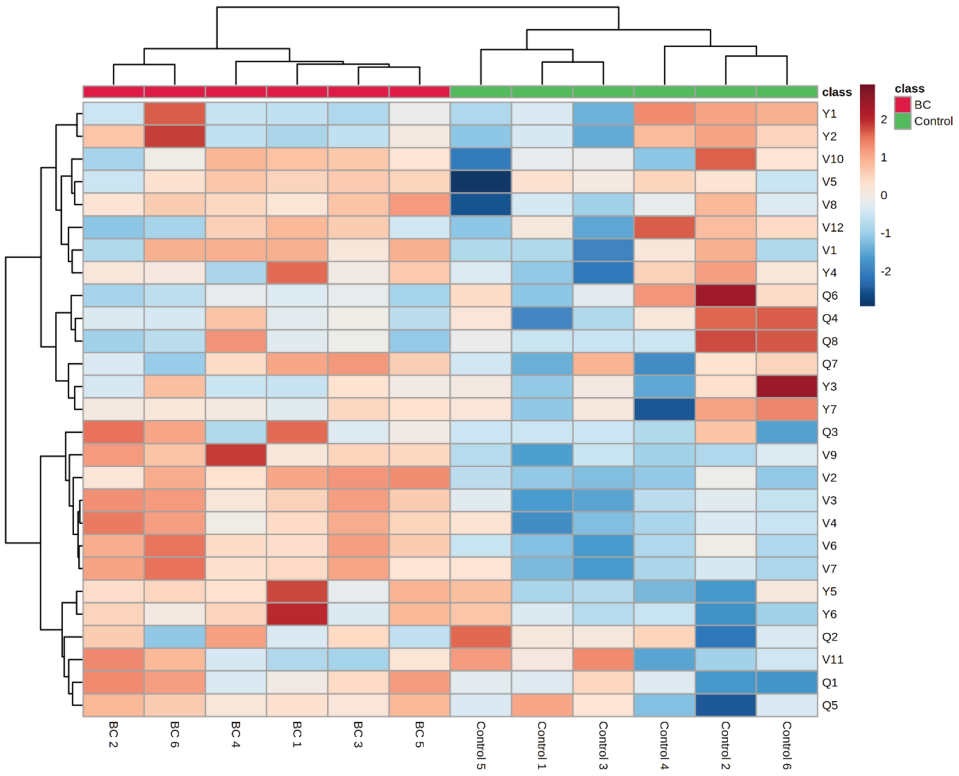

Fig. S1 - Heatmap of all vegetative, yield, and quality measures on strawberry plants

Table S5 - Measure code descriptors for heatmap (Fig. S1)

Code	Measure	Code	Measure
Q1	Total polyphenolics(GAE g · g^{-1}d.w.)	V7	Aerial (non-leaf) dry weight (g d.w.)
Q2	Brix of crude extract (°Bx)	V8	Aerial (non-leaf) water (%)
Q3	pH of crude extraxt (1/1000 dilution in water)	V9	Canopy area (cm^2)
Q4	Fruit dry weight (g d.w.)	V10	Leaf colour (*L**)
Q5	Fruit water %	V11	Leaf colour (*a**)
Q6	Fruit solids %	V12	Leaf colour (*b**)
Q7	Fruit extract (g·g^{-1} d.w.)	Y1	Unripe fruit (g)
Q8	Total flavonoids (QE mg · g^{-1}d.w.)	Y2	Total unripe fruit (number ·$plant^{-1}$)
V1	Number of crowns	Y3	Fruit fresh weight (g f.w.)
V2	Number of leaves	Y4	Harvest timing (days)
V3	Leaf fresh weight (gf.w.)	Y5	Total fruit harvested (g · $plant^{-1}$)
V4	Leaf dry weight (gd.w.)	Y6	Total fruit harvested (number · $plant^{-1}$)
V5	Leaf water (%)	Y7	Fruit quality compliance (% fruit count with FW > 10
V6	Aerial (non-leaf) fresh weight (g f.w.)		

Table S6 - Impact of BC on strawberry sensory perception

Measure	Average score		P
	Control	BC	
Aroma - Desirability	5.000	6.667	0.067
Taste - Desirability	4.500	5.170	0.555
Mouthfeel - Firmness	4.167	5.500	0.043
Mouthfeel – Juiciness	4.830	4.167	0.465
Aroma – Intensity	3.500	5.833	0.040
Taste - Sweet	4.000	3.833	0.822
Taste - Sour/acid	4.170	4.167	1.000
Taste - Intensity	4.670	4.500	0.899

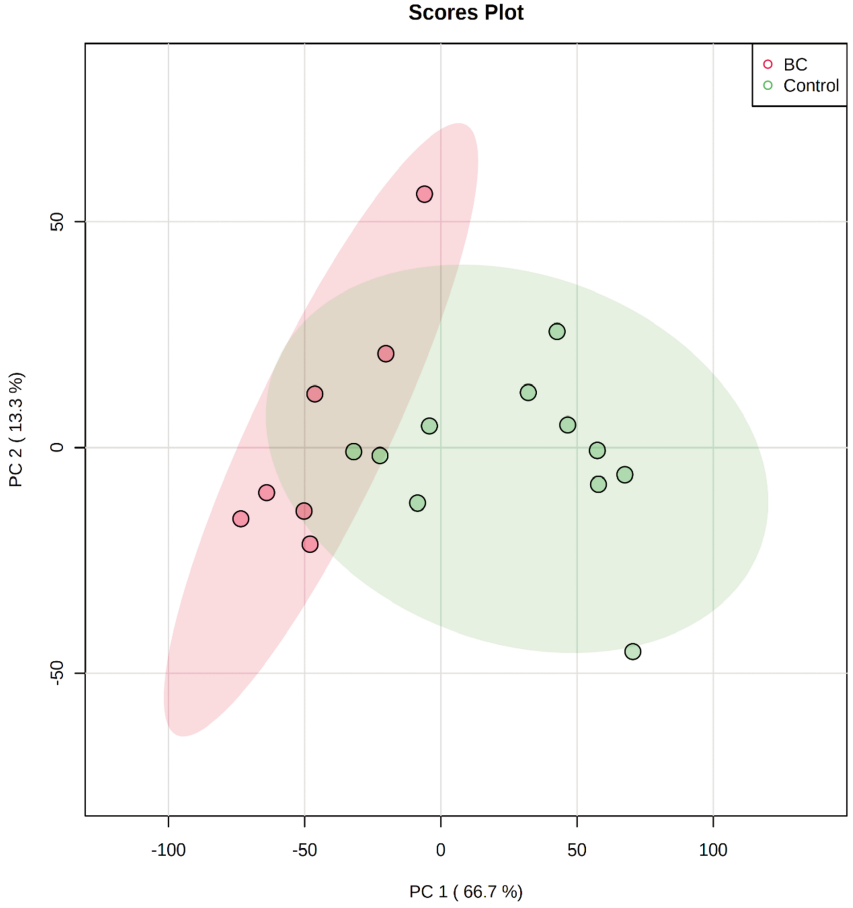

Fig. S2 - Principal component analysis (PCA) of M-IR spectra with shading indicating 95% confidence region.

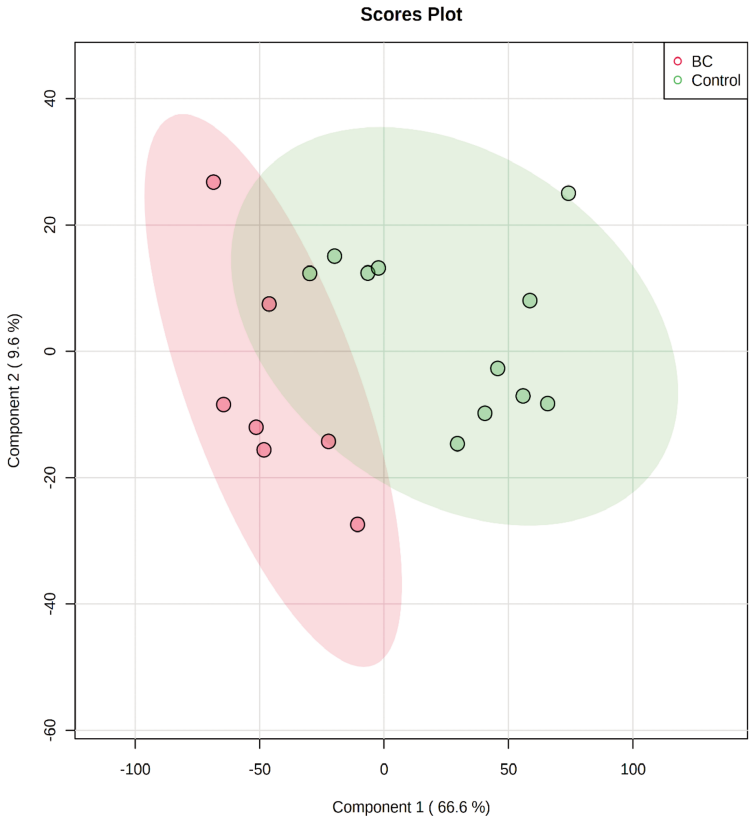

Fig. S3 - Partial least squares-discriminant analysis (PLS-DA) of M-IR spectra with shading indicating 95% confidence regions.

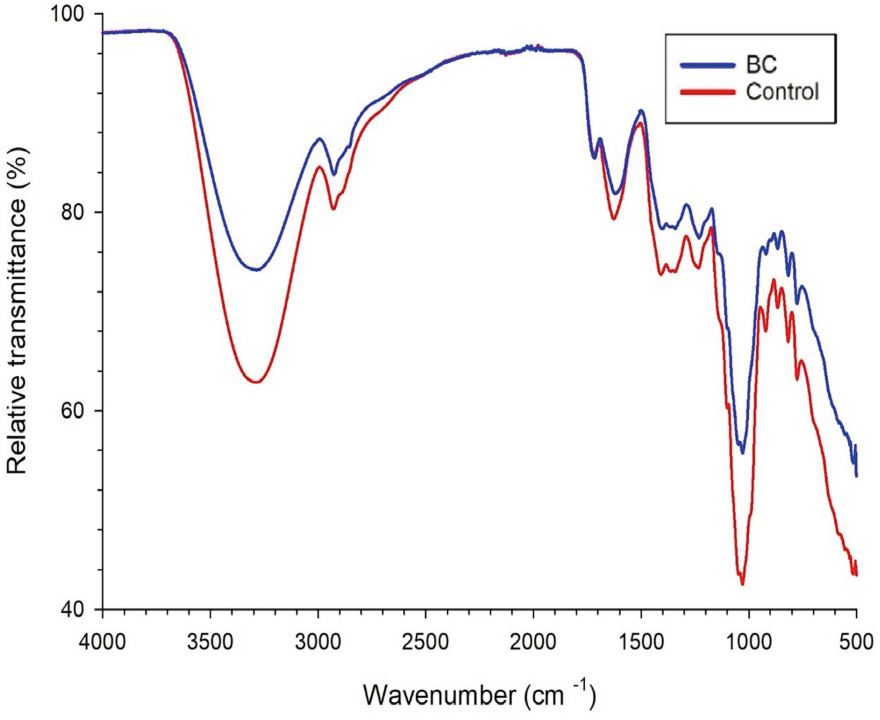

Fig. S4 - Mid-infrared (M-IR) spectra of dehydrated strawberry fruits analysed between 500–4000 cm-1 equipped with a Universal Attenuated Total Reflectance (UATR) accessory with diamond crystal. Spectra represent average absorbance of 7 and 11 biological replicates for control and BC, respectively.

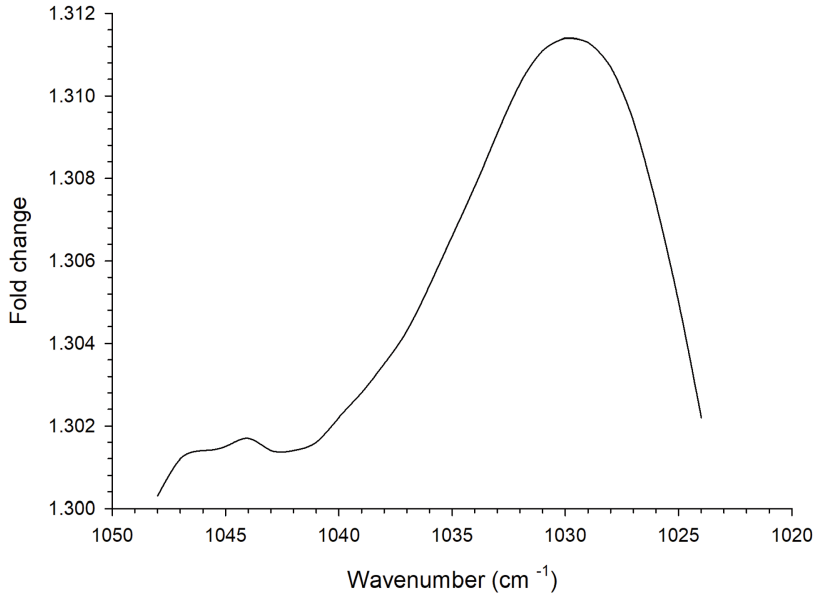

Fig. S5 - Fold change (> 1.3) in absorbance of average M-IR spectra for BC relative to average M-IRspectra for control.

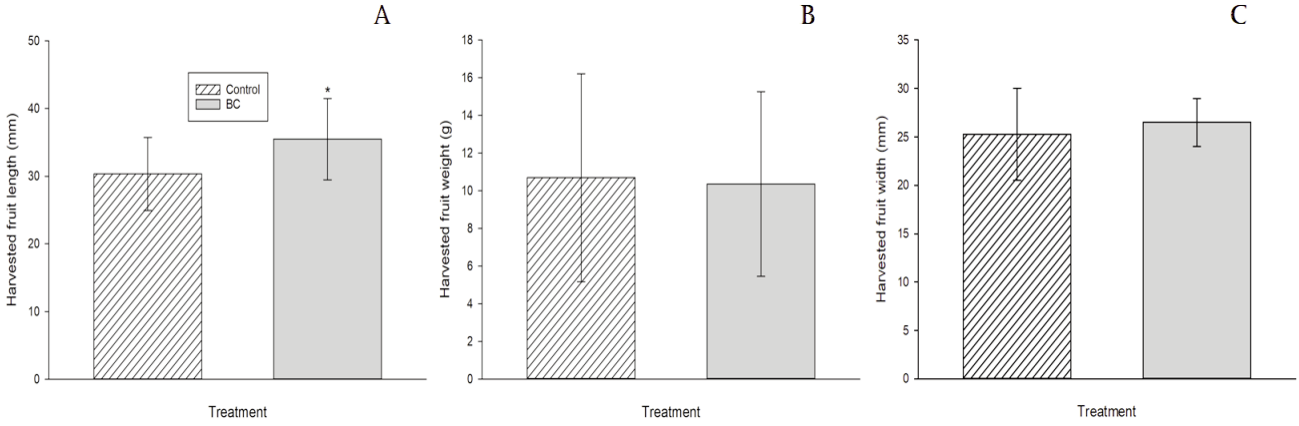

Fig. S6 - Effect of biostimulant complex (BC) on strawberry (*Fragaria x ananassa* 'Albion') fruit size parameters. A) length (control n = 15, BC n = 21), B) weight (control n = 10, BC = 12), and C) width (control n = 72, BC n = 112). Significant differences are indicated by * for $p < 0.05$ calculated by Student's t-test.

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In vitro salt stress tolerance of 'Sahand' cultivar grafted on two wild almond rootstocks: An evaluation of physiological and biochemical traits between rootstocks

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Keywords: Antioxidant enzymes, glycine betaine, grafting combination, proline.

Abstract: The low salinity tolerance of almond cultivars can cause a significant setback in almond production. Therefore, selecting suitable cultivars and rootstocks in salinity-affected areas can facilitate sustainable crop production. In this research, the effects of two wild almond species, Badamkohiand Arjan as rootstocks on the salinity tolerance of 'Sahand' as a scion were investigated through *in vitro* culture. A factorial experiment of 2 (species) × 4 (levels of salinity) was conducted in a completely randomized design (CRD) with 4 replications. The results showed that 'Sahand' grafted on Badamkohi had the higher fresh and dry weight than grafted on Arjan in all level of salinity. The Na^+ and Cl^- ions contents inthe shoots and root of both micrografting combinations increased with increasing salinity. However, their amount in the shoot and the root of 'Sahand'/Arjan plants were significantly higher than those ions in 'Sahand'/Badamkohi plants at 80 and 120 mM NaCl. The amount of total chlorophyll in 'Sahand' grafted on Badamkohi was 0.68 mg g^{-1} FW which was significantly higher than the total chlorophyll of the same scion grafted on Arjan rootstock (0.51 mg g^{-1} FW) at 120 mM NaCl. The highest leaf cell electrical leakage occurred in 'Sahand' grafted on Arjan which was significantly higher than leaf electrical leakage of the same scion grafted on Badamkohi at 120 mM NaCl. The grafting combination of 'Sahand'/Badamkohi showed a higher proline and glycine betaine content, compared to the grafting combination of 'Sahand'/Arjan. The shoot and root antioxidant enzyme activities (SOD, POX and CAT) in micrografting combination of 'Sahand'/ Badamkohi were also significantly higher than those in 'Sahand'/Arjan. It can be concluded that 'Sahand/Badamkohi combination is a suitable choice for the regions with late spring frost and saline conditions.

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Data Availability Statement:
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

Almond (*Prunus dulcis* Mill.) is one of the most important nut crops (Ansari and Gharaghan, 2019), with particular importance in the world. It

is characterized by a high storage capacity, low degree of waste, ease of processing and transportation. The feasibility of its economic production has led to an increase in the total area of almond orchards (Bybordi, 2013). Salinity still remains the major abiotic stress that limits agricultural production (Seleiman *et al.,* 2020). Almonds can be affected by salinity stress through osmotic mechanisms and by enhanced levels of osmotic potential in the soil solution (Shrivastava and Kumar, 2015). High salinity in root zone not only reduces water uptake and tree growth, but also can cause nutritional imbalances and toxicity effects of the major saline ions (Na^+ and Cl^-).

Meanwhile, the low salinity tolerance of almond trees can cause a significant setback in almond production (Kaundal *et al.*, 2019). Therefore, selecting suitable cultivars and rootstocks in salinity-affected areas can facilitate sustainable crop production.

The dynamic nature of salinity with respect to time and space, as well as limited experimental designs restrict the complete study of genotype-environment interactions (Sauvage *et al*., 2014). Therefore, the crop breeding program can be complemented with a suitable management option, such as grafting on appropriate salt tolerant rootstocks (Cuartero *et al*., 2006). Grafting has been reported as a rapid method for enhancing salt tolerance (Singh *et al*., 2020) that counteracts the salinity effects by maintaining low Na^+/K^+ ratios in the shoot and improves leaf stomatal conductance (Wei *et al*., 2017). The behavior of the rootstock in different plant species influences the metabolic processes of the scion leading to tolerance. Rootstocks are an essential component in modern fruit production (Shahkoomahally *et al*., 2020) and can provide several traits that may be absent in the scion, such as resistance to soil pest and disease, better root systems, enhanced nutritional uptake, better tolerance to soil salinity and water scarcity (Kumar *et al*., 2017).

The cultivation of grafted plants has gradually increased in recent years since grafting enables the plant system to control important agronomic traits and offers a flexible pattern to the growth of a particular scion (Kumar *et al.*, 2017). Grafting a scion on a suitable rootstock generally allows extensive use of the rootstocks (Gainza *et al*., 2015). While considering a wide range of salt-tolerant genotypes in the genus *Prunus*, the selection of more tolerant species as rootstock can lead to sustainable solutions in handling commercial cultivars of almond and peach (Najafian *et al*., 2008). The use of wild almond species as rootstocks, has been considered feasible especially in arid and semi-arid regions (Karimi *et al*., 2015). Using these rootstocks can highlight the strategy of allowing plants to overcome environmental stress, because of their adaptability and stimulated growth. Moreover, rootstocks affect the nutritional status of the scion and plant height (Aras and Eşitken, 2019).

Trees have lengthy biological cycles, which implies prolonged time lapses until plants are produced for study, as well as evaluations that can last for the entire growing season or even more than one season (Bado *et al*., 2015). This is of particular importance in plant breeding programs where thousands of plants are handled yearly and the staff and land are restricted. However, the long juvenile periods and cost of maintaining all the seedlings until they are grown trees have encouraged researchers to develop early studies in young plants to discard those genotypes which do not fit in the breeding goals and minimize the cost and time of field evaluations (Vives-Peris *et al*., 2017).

In recent years, tissue culture and *in vitro* selection have emerged as an effective tool in the furtherance of efforts to develop stress-tolerant plants. *In vitro* cultivation techniques can largely assist with the study and selection of plant species, because more control is exerted on plant growth compared to the outside environment, and evaluations are usually conducive to good results in a confined space (Ghaleb *et al*., 2010; Rai *et al*., 2011). When the stability of medium culture enables a controlled condition, a uniform application of stress to all explants can create reliable results, because other intervene factors are eliminated (Seth and Kendurkar, 2015). The *in vitro* system can characterize the degree of salt tolerance of different genotypes at their primary growth phase within a short time, limited space and low cost (Ghaleb *et al*., 2010). This method has been applied for screening salt tolerant genotypes of some fruit species including cherry rootstocks (Erturk *et al*., 2007), citrus rootstocks (Ghaleb *et al*., 2010), grape rootstocks (Alizadeh *et al.,* 2010), fig cultivars (Abdoli Nejad and Shekafandeh, 2014), apple varieties (Shibli *et al.,* 2000), kiwifruit (Sotiropoulos and Dimassi, 2004) and pear (Sotiropoulos *et al*., 2006). The aim of this research was to study the influence of Badamkohi and Arjan as rootstocks on the salinity tolerance of 'Sahand' (a late-bloom almond cultivar) as a scion through certain morphological and bio-

chemical responses *in vitro* condition.

2. Materials and Methods

Establishment of in vitro *micrografting*

According to Asadi and Shekafandeh (2021) procedure, the mature seeds of naturally grown wild almond trees, *Prunus scoparia* (C. Schneider) and *Prunus elaengnifolia* (E. Murrary) named Badamkohi and Arjun respectively, grow in arid and semi-arid regions were grown *in vitro* to produce seedlings. After removing the endocarps, they were surface-sterilized by immersion in 70% alcohol for 1 min and then in 20% Whitex solution (sodium hypochlorite 5%) for 10 min. Subsequently, they were rinsed three times with sterile distilled water. The sterilized seeds were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar as gelling agent (Fig. 1 a, b). For preparation of the micro-scion, young shoots of the late-blooming 'Sahand' were disinfected and cultured on MS medium supplemented with 2.2 Mm benzyladenine (BA) and 0.54 µM naphthaleneacetic acid (NAA). After two weeks, the young offshoots have reached a suitable size to be used as scions (Fig. 1 c). Then, the two weeks old *in vitro* produced seedlings were decapitated and by the help of a sharp scalpel a vertical slit (0.5 cm) was created on top of the stump (Fig. 1 d). The scion was cut into a "V" shaped wedge (Fig. 1 e) and inserted into the rootstock to form a micrograft (Fig. 1 f).

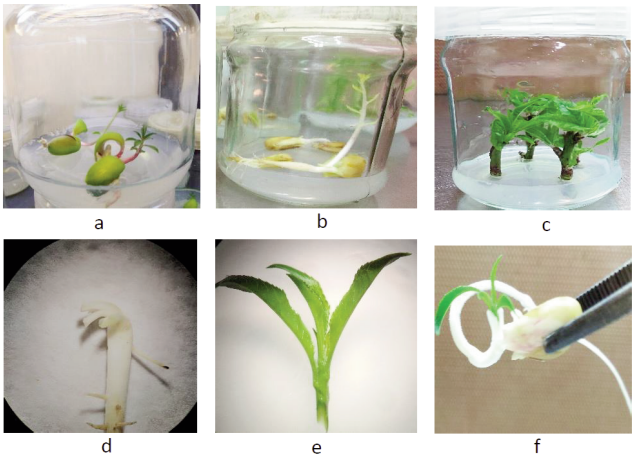

Fig. 1 - Performing *in vitro* micrograft. Germinated seeds of Arjan (a) and Badamkohi (b) using as rootstock. Proliferated shoots of 'Sahand' cv. on MS medium supplemented with 2.2 µM BA and 0.54 µM NAA using as scion (c). An Arjan rootstock ready to be grafted (d). A scion ready for grafting (e). A micrograft combination of 'Sahand'/Arjan (f).

The jars containing explants were maintained at 25±1°C under a 16 h photoperiod (light intensity of 4000 lux).

Salinity treatments

The graft combinations were allowed to grow for 4 weeks, and then they were cultured on agar free MS medium with a bridge paper and different concentrations of NaCl (0, 40, 80 and 120 mM). After 4 weeks of culture, certain morpho-physiological and biochemical characteristics of graft combinations were measured as follow.

Fresh and dry weight of scion and rootstock

After measuring the fresh weight of the shoot (scion) and the root (rootstock), they were dried in an oven for 24 h at 60°C and then re-measured for their dry weight (in mg).

Sodium (Na^+) and Chlorine (Cl^-)

The samples (1 g) were dried in an oven at 500 to 550°C for 5 h, then reduced to ash. To each sample in the crucible was added 5 ml of 2 normal HCl. After passing the solution through filter paper, the filtered solution was transferred to a jug balloon. The volume of each sample was made up to 50 ml with hot distilled water and then sodium was measured using a flame photometer (Model Jenway PFP7 Bibby Scientific Ltd, Staffordshire, UK) and calculated in mg g^{-1} dry weight.

To determine the chlorine of each sample, according to the method of Chapman and Pratt (1961), one gram of sample was poured into a Chinese mortar and 250 mg of calcium oxide was added to each and was kneaded with distilled water. They were then placed in a kiln at 250°C for one h to remove all the soot from the initial burning. After, the kiln temperature was slowly raised to 550°C to reduce the samples to ashes. Then 15 mL of hot distilled water was added to the samples. After cooling, 5 drops of 5% potassium chromate were added to the solution and titrated with 0.05 N silver nitrate (2.12 g of silver nitrate in 250 mL of distilled water) to observe a red brick-colored precipitate. Finally, chlorine was calculated as mg g^{-1} dry weight.

Total chlorophyll (Chl)

Fresh leaf samples (0.1 g) were placed in test tubes and added 7 mL of dimethyl sulfoxide, then they were placed in an incubator for 30 minutes at 65°C. After extraction, the volume of extracts was made up to 10 mL by adding dimethyl sulfoxide.

Finally, the absorbance of the extracts at wavelengths of 645 and 663 nm was read using a spectrophotometer (USA Epoch Microplate, BioTek instruments, Inc) (Gross, 1991). Chlorophyll content was determined as follows:

$$\text{Chl (mg g}^{-1}\text{) FW} = [20.2\ (OD645nm) + 8.02\ (OD663nm)] \times V/FW \times 1000$$

Where V is final volume of solution (mL), FW the leaf fresh weight (mg), and OD the optical density.

Electrolyte leakage(EL)

EL was determined according to the method of Gulen and Eris (2004). Ten discs were cut from the fully developed leaves of the plants in each replication. Then, they were transferred to vials containing 5 mL deionized water and kept at 10°C for 24 h. After measuring their electrical conductivity (EC1) using a conductometer (Metrohm 644, Awess), the samples were then placed in a water bath at 95°C for 20 min, and after cooling at 25°C the electrical conductivity (EC2) was re-measured. The EL was calculated with the following formula:

$$EL\ (\%) = (EC1/EC2) \times 100$$

Proline contents

According to the modified method of Bates (1973), the proline content was determined. The leaf sample (0.5 g) was grinded in 10 mL sulfosalicylic acid (3%). The mixture was then centrifuged at 10,000×*g* for 10 min. Two ml of the supernatant was added to each test tube which contained freshly prepared acid-ninhydrinas a diluted solution (2 ml). The tubes were incubated in a water bath at 90°C for 30 min. Ultimately, the reaction ended in an ice bath. The reaction mixture was extracted using toluene (5 mL) and was vortexed for 15 s. The tubes were stored in darkness at room temperature for 20 min, thereby allowing the separation of toluene from the aqueous phase. The toluene phase was then carefully collected and the absorbance was measured at 520 nm by a spectrophotometer (model T60 USA).

Glycine betaine

Glycine betaine was measured by the method of Grattan and Griere (1985). The powdered sample of leaf (0.5 g) was mixed in a mortar with 20 mL of ionized water. The specimens were placed on a shaker for 48 h at 25°C. They were passed through a Whatman filter paper 'G42' and diluted in a 1:1 ratio with two-molar sulfuric acid. Then, 0.5 mL of this solution was removed and poured into the Eppendorf tube. After cooling the samples for 2 h, 0.2 ml of potassium tri-iodide solution was added to each. Then, they were centrifuged for 20 min at 15,000 rpm at 0°C. The top phase discarded and the periodontal crystals were dissolved in the bottom of the container in 9 mL of 1-2 dichloroethane. Then, the absorption of the samples was measured at 365 nm with a spectrophotometer.

Enzyme activity

In order to estimate the enzymes activities, the leaf samples (0.5 g) were first homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 3 mM 2-mercaptoethanol, and 2% (w/v) polyvinyl polypyrrolidone (PVPP) in a chilled mortar. The homogenate was then centrifuged at 16000 *g* for 30 min at 4°C and the supernatant was used for enzyme assays.

Superoxide dismutase (SOD)

The SOD (EC 1.15.1.1) can be measured by determining its ability to halt the photochemical reduction of nitro blue tetrazolium chloride (NBT) in the presence of light. In this method, the reaction mixture (3 mL) contained 50 µL enzyme extract, 50 mM potassium phosphate buffer, 13 mM l-methionine, 75 µM NBT, 0.1 mM EDTA and 4 µM riboflavin. The reaction mixture was shaken and placed in a light chamber for 15 min to allow the reaction to take place. Eventually, the absorption rate of each specimen was recorded at 560 nm using a spectrophotometer (Biochrom WPA Biowave II UV/Visible Spectrophotometer, England) against the non-irradiated blank (Dhindsa and Motowe, 1981).

Catalase (CAT)

The determination of the activities of CAT (EC 1.11.1.6) was based on the rate of H_2O_2 decomposition as measured by decreasing the absorbance at 240 nm (Dhindsa and Motowe, 1981). While the reaction mixture contained 50 mL potassium phosphate buffer (pH 7) and 15 mM hydrogen peroxide (H_2O_2), the reaction started by adding 1000 µL of the enzyme extract. One unit of activity is the amount of enzyme that could decompose 1 mM of H_2O_2 in 1 min.

Peroxidase (POX)

Peroxidase enzyme activity was read at 470 nm, based on an enhanced degree of light absorption as a result ofguaiacol oxidation in the presence of peroxidase hydrogen. This was carried out by a spectropho-

tometer (JENWAY model 7315 UK) in 1 min with a time interval of 10 s (Ozden *et al*., 2009). The activity of the enzyme was calculated based on the oxidized µmol of guaiacol per min and per g of fresh leaf weight.

Statistical analyses

The experiment was carried out as a factorial 2 (species) × 4 (levels of salinity) in a completely randomized design (CRD) with 4 replications and 4 micrografted plants per replicate. A total of 122 micro-grafting combination of 'Sahand'/Argan and 'Sahand'/Badamkohi were used in this experiment. Data were analyzed using SAS 9.4 software and mean values were compared using LSD test (P≤0.05).

3. Results

The results of analysis of variance showed that the interaction between species and salinity was significant at 5% or 1% level in all measured traits. So, all the results were presented by the interactions.

Effects of salinity on scion-rootstock combination growth

The results showed that, both rootstocks (Badamkohi and Argan) had significant difference in the root length of control (free salt medium). Badamkohi showed the highest root length of 263 mm that was significantly higher than Arjan (122 mm) (Table 1).

This showed that they have different growth habit. However, in both rootstocks with increasing salinity to 120 mM in culture medium the root length decreased significantly. This reduction was 50% and 18% for Badamkohi and Arjan respectively compared to their controls. The highest length of scion (64 mm) was related to the salt-free treatment on Badamkohi rootstock which showed a significant difference with the same scion on Arjan rootstock (41 mm) in the same treatment.

With increasing the concentration of sodium chloride from 0 (control) to 120 mM, the scion ('Sahand') length on both rootstocks was significantly reduced (Fig. 2 b, c, e, f), this reduction was 50.6% and 49% on Badamkohi and Arjan respectively.

Table 1 - Effect of sodium chloride on scion and root length in micrografting combinations of 'Sahand'/Badamkohi and 'Sahand' /Arjan

Micrografting combination	NaCl mM	Root length (mm)	Shoot length (mm)
'Sahand'/Badam kohi	0	263 a	64 a
	40	202 b	44 b
	80	148 c	38 bc
	120	130 d	30 d
'Sahand'/Arjan	0	122 ed	41 b
	40	114 edf	33 cd
	80	106 ef	29 d
	120	100 f	21 e

In each column, means with the same letters are not significantly different at 5% probability level using LSD test.

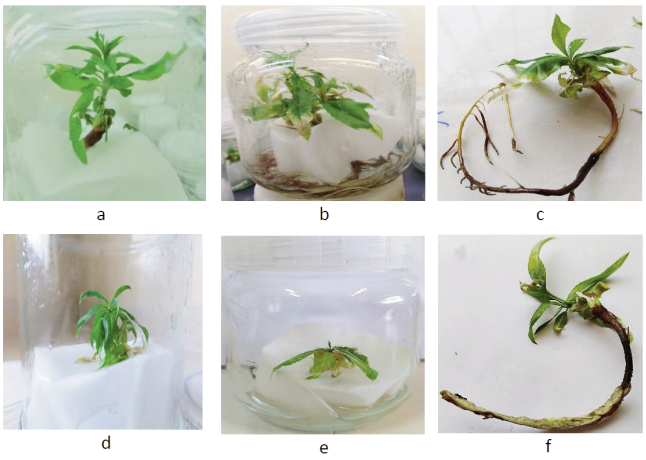

Fig. 2 - Growth of a micrografted combination of 'Sahand'/Badamkohi in control (a) and in 120 mM salinity (b and c); a micrografted combination of 'Sahand'/Arjan in control, (d) and 120 mM salinity (e and f).

In both rootstocks, root fresh and dry weight decreased with increasing NaCl concentrations, however fresh weight loss in Badmkohi and Arjan was 62% and 76%, respectively, and also dry weight loss was 55% in Badamkohi and 51% in Arjan (Fig. 3 a and c). Fresh and dry weight of 'Sahand' grafted on Badamkohi at all salinity levels (except scion dry weight in 120 mM salt) was significantly higher than fresh and dry weight of 'Sahand' grafted on Arjan (Fig. 3 b and d). In both rootstocks, increasing the salt from 0 to 120 mM reduced the fresh weight of the scion by about 60% while this reduction in the dry weight of scion was 62% on Badamkohi rootstock and 55% on Arjan rootstock (Fig. 3 b and d).

Effect of salinity on Na^+ and Cl^- contents in micrografting combinations

The results showed that the Na^+ and Cl^- contents in the roots and the shoots of both micrografting combinations increased with increasing salinity (Fig. 4 a, b, c, d,). However, the amount of Na^+ and Cl^- in the shoot and the root of 'Sahand'/Arjan plants were sig-

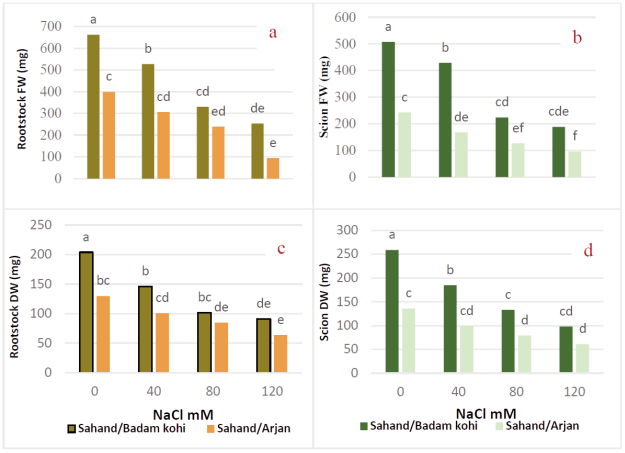

Fig. 3 - The effect of different concentrations of NaCl on fresh and dry weight of micrografting combinations of 'Sahand'/ Badamkohi and 'Sahand'/Arjan. Different letters indicate significant difference at P ≤ 0.05 level of probability using LSD test.

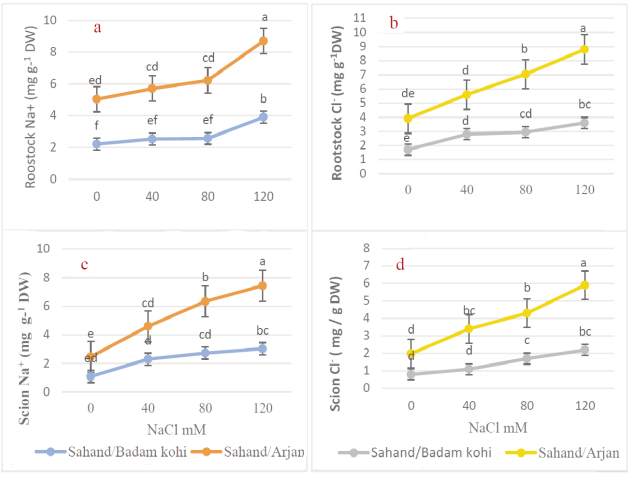

Fig. 4 - The Na+ and Cl- contents of both rootstocks (a, b) and scion (c, d) in grafting combinations of 'Sahand' /Badamkohi and 'Sahand'/Arjan in different concentration of NaCl. Different letters indicate significant difference at P ≤ 0.05 level of probability using LSD test.

nificantly higher than these ions in the shoot and the root of 'Sahand'/Badamkohi plants at 80 and 120 mM NaCl.

Total chlorophyll (Chl)

The Chl of 'Sahand' grafted on both rootstocks decreased with increasing salt concentration. The highest amount of Chl was obtained in 'Sahand' (as scion) leaf grafted on Badamkohi rootstock in unsalted medium (control) which showed a significant difference compared to the same scion grafted on Arjan rootstock. The amount of Chlin 'Sahand' grafted on Badamkohi was 0.68 mg g^{-1} FW which was significantly higher than the Chl of the same scion grafted on Arjan rootstock (0.51 mg g^{-1} FW) at 120 mM NaCl (Table 2). At the highest salinity level, the leaf chlorophyll content of 'Sahand' either grafted on Badamkohior Arjan was decreased by 51% and 63% respectively compared to their controls.

Electrolyte leakage (EL)

In all levels of salinity, the EL of 'Sahand' grafted on Arjan rootstock was significantly greater than the same cultivar grafted on Badamkohi. The highest leaf cell EL (72.29%) occurred in 'Sahand' grafted on Arjan rootstock which was significantly higher than leaf EL of the same scion grafted on Badamkohi rootstock at 120 mM NaCl (Table 2).

Table 2 - Effects of sodium chloride on leaf total chlorophyll and electrolyte leakage in micrografting combinations

Micrografting combination	NaCl mM	Total chlorophyll (Mg g^{-1} FW)	Electrolyte leakage (%)
'Sahand'/Badam kohi	0	1.45 a	23.28 e
	40	1.42 ab	31.32 de
	80	0.95 bc	38.05 cd
	120	0.68 d	43.86 c
'Sahand'/Arjan	0	1.33 b	38.15 cd
	40	1.25 bc	43.77 c
	80	0.77 d	55.15 b
	120	0.51 e	72.29 a

In each column, means with the same letters are not significantly different at 5% probability level using LSD test.

Proline and glycine betaine (GB) in micrografting combination

Increasing the level of salinity caused a significant rise in the proline content of both grafting combinations ('Sahand'/Badamkohi and 'Sahand'/Arjan) (Table 3). The grafting combination of 'Sahand'/ Badamkohishowed a higher proline content (in both parts, scion and rootstock) compared to the grafting combination of 'Sahand'/Arjan in all salinity levels.

The results also indicated that by increasing the salt concentration from 0 to 120 mM, the amount of GB increased in both micrografting combinations. The shoot and the root in micrografting combination of 'Sahand'/Badamkohi showed significantly greater GB than those in micrografting combination of 'Sahand'/Arjan in all level of salinity (except for root GB in salt free medium of both rootstocks).

Table 3 - Effects of sodium chloride on the amounts of proline and glycine betaine (GB) in the shoots and roots of micrografting combinations

Micrografting combination	NaCl levels (mM)	Root GB µmol g^{-1} DW	Shoot GB µmol g^{-1} DW	Root proline µmol g^{-1} FW	Shoot proline µmol g^{-1} FW
'Sahand'/Bdam kohi	0	10.55 ef	6.88 cd	24.93 e	20.64 d
	40	14.60 d	7.48 c	38.03 c	27.73 c
	80	19.00 b	8.90 b	46.70 b	40.88 b
	120	22.60 a	11.00 a	62.40 a	55.38 a
'Sahand'/Arjan	0	9.30 f	5.70 e	14.80 f	13.22 e
	40	11.47 e	6.30 de	21.70 ef	18.97 de
	80	14.02 d	6.80 cd	28.9 de	24.06 cd
	120	16.50 c	7.60 c	34.50 cd	30.40 c

In each column, means with the same letters are not significantly different at 5% probability level using LSD test.

Superoxide dismutase (SOD)

SOD activity in both'Sahand'/Badamkohi and 'Sahand/Arjan plants increased with increasing the levels of salinity (Table 4). The highest increase in the activity of SOD was obtained in the 'Sahand' /Badamkohi, combination with 101.27 and 103.30 (U g^{-1} FW min^{-1}) in shoot and root respectively at 120 mM salt, which was significantly higher than those in 'Sahand/Arjan plants.

Peroxidase (POX)

In the shoots and in the roots of both micrograft combinations, POX activity increased with increasing salt concentrations in the medium. Although, at all salinity levels, POX activity was higher in the shoot and the root of micrografting combination of 'Sahand'/Bada Kohi than 'Sahand'/Ajan, however the difference was only significant at 120 mM NaCl.

Catalase (CAT)

Both micrografting combinations showed the enhancement CAT activity in response to increasesalt concentrationfrom 0 to 120 mM NaCl. The highest CAT activity occurred in the shoot (63.70 U g^{-1} FW min^{-1}) androots (59.70 U g^{-1} FW min^{-1}) of 'Sahand'/ Badamkohi plants at 120 mM NaCl which was significantly higher than the activity of this enzyme in the 'Sahand'/Arjan plants (Table 4).

Table 4 - Effects of sodium chloride on the shoot and root enzymes activities (SOD, POX and CAT) in micrografting combinations of 'Sahand'/ Badamkohi and 'Sahand'/Arjan

Micrografting combination	NaCl (mM)	SOD shoot U g^{-1} FW min^{-1}	SOD root U g^{-1} FW min^{-1}	POX U g^{-1} FW min^{-1}shoot	POX root U g^{-1} FW min^{-1}	CAT shoot U g^{-1} FW min^{-1}	CAT root U g^{-1} FW min^{-1}
'Sahand'/Badam kohi	0	90.10 e	76.60 c	31.62 ed	51.60 fe	40.60 cd	32.50 ef
	40	92.90 d	77.30 bc	48.81 bc	60.30 cde	41.80 cd	44.90 bc
	80	96.10 c	80.00 bc	50.70 b	69.80 bc	46.90 bc	48.70 b
	120	101.27 a	103.30 a	76.99 a	95.00 a	63.70 a	59.70 a
'Sahand'/Arjan	0	87.60 f	72.10 c	23.40 e	48.10 f	39.60 d	31.30 f
	40	92.20 d	75.20 c	36.60 cd	58.80 de	41.10 cd	38.60 de
	80	95.20 c	78.00 c	45.60 bc	67.20 bcd	43.60 bcd	42.40 cd
	120	97.60 b	85.60 b	47.80 bc	73.50 b	50.50 b	46.20 bc

In each column, means with the same letters are not significantly different at 5% probability level using LSD test.

4. Discussion and Conclusions

Performance of grafted plants compared to non-grafted or self-grafted plants under a stressful condition is often dependent on the rootstock's root system characteristics. A vigorous root system could be the most important criterion for increasing salt tolerance (Balliu *et al*., 2007). In this research, Badamkohi showed the highest root length of 263 mm that was significantly higher than Arjan (122 mm). The rootstock's root systems architecture specified by root length and density, root hairs and root surface area play a critical role in ion and water uptake, thus determining salt tolerance of grafted plants (Colla *et al*., 2010). A vigorous root system, for instance, produced more cytokinins and transported water to the shoot system by xylem sap, which positively affected plant growth and crop yield (Oztekin and Tuzel, 2011). Furthermore, hydraulic conductivity of the roots may control plant growth by manipulation of the water supply to epigeous plant parts (Gregory *et al*., 2013).

The most immediate effect of salinity on plants is the inhibition of root and shoot development. Due to the imbalance of water potential between the apoplast and simplast, the osmotic potential decreases and the absorption of water is hampered. Ultimately, this reduces plant growth by closing the stomata and weakening photosynthesis (Dustgeer *et al*., 2021). Fresh and dry weight of plants decrease with increasing salinity, which is usually due to ion toxicity and water stress (Arif *et al.*, 2020; Corell *et al*., 2020). Salinity has the effects on metabolic activity and reduces the division of new cells and disrupt cellular processes, thus reduces plant growth compared to normal conditions (Carillo *et al*., 2019). In this study, as mentioned before, the root system of Badamkohi was stronger than Arjan. However, in high level of salinity, the shoot length of the same scion ('Sahand') on Badamkohi was greater than on Arjan. It seems that the Arjan rootstock has a dwarfing effect on the scion. On the other hand, at all salinity levels, the fresh and dry weight of the scion on Badamkohi was more pronounced than on Arjan. This means that Badamkohi supports scion growth better than Arjan.

It has been also reported some rootstocks are more capable of inducing tolerance to the scion against salt stress (Zrig *et al*., 2016; Aras and Eşitken, 2018). In this regard, adding sodium chloride to the growth medium of two graft combinations ('Sahand'/Badamkohi and 'Sahand'/Arjan) led to a decrease in scion growth, however this growth reduction of 'Sahand' subjected to increasing concentrations of NaCl was more acute when the rootstock was Arjan. This result is in consistence with the results of sweet almond grafted on different rootstocks (Zrig *et al.,* 2016).

Sodium ion (Na^+) is toxic to cellular metabolism and affects the activity of some enzymes, and high concentrations of Na^+ causes ion imbalance (Roy *et al.,* 2014).

The ability of almond rootstocks varies in absorbing or transferring sodium to the scion and there is a very close relationship between tolerance to salinity and the amount of sodium transferred to the leaves (Mickelbart and Arpaia, 2002). In the present research, Na^+ concentration in the aerial parts was lower in 'Sahand'/ Badamkohi compared to 'Sahand'/Arjan. Such a prohibiting mechanism may explain, the higher shoot length and biomass of 'Sahand'/Badamkohithat observed in our study. A higher Na^+ concentration in the environment of root can depress K^+/Na^+ ratios in the plant, thereby, the plant becomes susceptible to specific ion injury as well as to nutritional disorders which may affect growth and yield. The exclusion of Cl^- from shoots is related to the ability of cell membranes to restrict the movement of Cl^- through the root to vascular tissue and the degree of Cl^- accumulation in the roots (Walker and Douglas, 1983). In this experiment, Badamkohi rootstock was able to slow the accumulation of Cl^- in the leaves. Similar results have been reported by García-Sánchez *et al*. (2002) in which the Cleopatra rootstock reduced the accumulation of Cl^- in the scion compared to the Carrizo rootstock.

The finding of this experiment showed that Badamkohi rootstock hold up more the chloroplast integrity of 'Sahand' than Arjan rootstock. Chlorophyll depletion in salt stress can be linked to factors such as structural damage of chloroplasts due to the formation of reactive oxygen species and photo oxidation of chlorophyll (Taïbi *et al.,* 2016), the destruction of chlorophyll synthesis precursors, the inhibition of biosynthesis of new chlorophylls, and hormone disorders (Sabzmeydani *et al.,* 2020). This reduction could also be due to the increase in the activity of the enzyme chlorophyllase or to the instability of the protein pigment complexes by the ions (Saha *et al.,* 2010). Surendar *et al.* (2013) reported a decrease in chlorophyll content under stress was caused to the destruction of the chloroplast mem-

brane with increasing phosphatase activity, which is located on the membrane.

Plasma membranes are the primary site of ion-specific salt injury. Undesirable performance of the cell's metabolism during periods of abiotic stress leads to the stimulation of reactive oxygen species which would damage the cell membrane and increase electrolyte leakage. Therefore, electrolyte leakage from plasma membranes is reported as one of the most important selection criteria for identification of salt-tolerant plants (Besma and Denden, 2012). In this study, especially at higher levels of salinity, the 'Sahand' grafted on Arjan experienced a greater damage to the cell membrane of its leaves, than grafted on Badamkohi. This indicates Badamkohi rootstock's ability to maintain the integrity of scion cell membrane in salt stress conditions. In accordance with our finding, Colla *et al.* (2012) reported that in cucumber grafted plants, the amount of ion leakage in salinity stress is reduced compared to non-grafted plants and rootstock helps the maintenance of membrane function.

In this study, the grafting combination of 'Sahand'/Badamkohi accumulated more proline and glycine betaine in the root and shoot, compared to the same scion grafted on Arjan. Proline accumulation in salinity condition can play a role in stress tolerance mechanismsby stabilizing proteins at high ionic concentrations (Krasensky and Jonak, 2012). It also reduces damages caused by salinity via the preservation of water in cells and by diluting salts in the plant (Gulen *et al*., 2018). Proline has essential functions by osmoregulation, reducing the undesirable effects of ROSs under salinity stress.Therefore, higher proline accumulation can induce higher tolerance against salinity by the plant (Akbari *et al.,* 2018). The proline content of micrografting combination significantly increased in response to an increase in the salinity level, in this regards 'Sahand'/Badamkohi micrograft combination was more prominent than 'Sahand'/Arjan. Our finding is in agreement with other reports on *in vitro* salt tolerance of pistachio (Raoufi *et al.*, 2020) and fig (Abdoli Nejad and Shekafandeh, 2014). Glycine betaine was also found to play significant roles in enhancing salt tolerance (Wei *et al*., 2017). It can maintain the osmotic regulation, improve the production, nutrients and water absorption, thereby photosynthetic proteins are produced and membrane peroxidation is reduced (Dustgeer *et al.,* 2021). In this research, the grafting combination of 'Sahand'/Badamkohi accumulated more glycine betaine in the root and shoot, compared to the grafting combination of 'Sahand'/Arjan, that means Badamkohi protects cell osmotic pressure, enhancing cell membrane integrity as well as maintaining photosynthetic apparatus (Niazian *et al*., 2021).

As mentioned above, with increasing salinity levels, Badamkohi rootstock enhanced SOD, POX and CAT activity in 'Sahand' Scion more than Arjan rootstock. SOD plays a major role in ROS scavenging in plants and is considered as the first line of defense against the toxic effects of elevated ROS (Hou *et al.,* 2019). SOD catalyzes the dismutation of superoxide radicals to H_2O_2 and O_2 (Feng *et al.,* 2015). Increasing the level of superoxide dismutase activity is important for protecting chloroplasts and mitochondria from the stress of reactive oxygen species. In fact, under stress conditions, the chloroplast is where the majority of active oxygen species are produced and where is caused the highest degree of damage (Sofo *et al.,* 2005; Kuşvuran *et al.,* 2016).

According to the results of the present study, salinity stress caused the SOD activity to increase significantly in both micrograft combinations ('Sahand'/Badamkohi and 'Sahand'/Arjan). Nonetheless, Badamkohi rootstock enhanced the SOD activity in 'Sahand' more than Arjan rootstock. Enhanced activities of SOD enzyme usually reflect defensive responses to cellular damage induced by higher NaCl concentrations in the culture medium (Akbari *et al.,* 2018; Kuşvuran *et al.,* 2021).

The enzyme hydrogen peroxidase (POX) also reduces oxidative stressby protecting the metabolic enhancers that sustain cell and plant survival (Aliakbarkhani *et al*, 2017). The results of this study showed that by increasing salinity levels in the growth medium of the two grafted combinations, there was an increase in the activity of peroxidase enzyme and this increment was more obvious in 'Sahand' grafted on Badamkohi rootstock. According to Fayek *et al*. (2018), the activity of POX enzyme changes with different scions graftedon different rootstocks. An increase in POX activity in grafted plants could be an indicator that the grafting process can rapidly induce a higher capacity to breakdown H_2O_2 in plant cells (Elsheery *et al*., 2020).

Catalase is one of the most important enzymes that can inhibit ROS activity. It converts hydrogen peroxide to water and oxygen in the mitochondria, peroxisomes and cytosol (Acosta-Motos *et al.,* 2017). Based on the results of this study, with increasing salinity levels, the activity of catalase increased in

both grafted combinations showing that tolerance to salinity corresponds with an increase in catalase enzyme of the plants, although Badamkohi rootstock was more tolerant than Arjan. The greater availability of CAT can enable the plant defense mechanism to increase the capability of eliminating reactive oxygen species (Madadkhah *et al.,* 2018). Our finding corresponds with those reported previously in pistachio (Akbari *et al.,* 2018) and cherry (Chatzissavvidis *et al.,* 2008).

In all levels of salinity 'Sahand'as scion grafted on Badamkohi had more shoot length, fresh and dry weight than grafted on Arjan. Badamkohi restricted the absorption of Na^+ and Cl^- ions from the root medium and reduced their transportation to aerial parts. The 'Sahand' leaf chlorophyll depletion and EL were higher on Arjan rootstock than on Badamkohi in all level of salinity condition. Badamkohi protected cell osmotic pressure, enhancing cell membrane integrity than Arjan by inducing more proline and GB as osmoprotectants in the shoots. Badamkohi rootstock also enhanced the activities of antioxidant enzymes in 'Sahand' more than Arjan rootstock which reflect defensive responses to cellular damage induced by reactive oxygene species in higher NaCl concentrations in the culture medium. In conclusion, Badamkohi could be a more suitable rootstock than Arjan for 'Sahand' scion under salinity conditions.

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Comparison quality parameters of saffron (*Crocus sativus* L.) produced in Herat, Afghanistan and Torbat Heydarieh, Iran

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Key words: Crocin, picrocrocin, safranal, secondary metabolites, spectrophotometric analysis.

Abstract: Saffron *Crocus sativus* L. (Iridaceae) is one of the most valuable and expensive medicinal plants in the world. In order to compare the quality characteristics of saffron in Afghanistan and Iran, samples of dried *C. sativus* from different saffron-producing regions of Herat province (Afghanistan) and Torbat Heydarieh county (Iran) were collected in the year 2021. The experiment was analyzed in GLM format and Nested method with three replications. The samples of saffron produced from seven different districts of Herat province in Afghanistan, including Injil, Karukh, Guzara, Pashtun Zarghun, Zendeh Jan, Ghoryan, Obeh and nine saffron-producing villages (Fakhrabad, Kadkan, Nasar, Ghaleno, Feizabad, Khorgh, Abrood, Benhang, Shadmehr) of Torbat Heydarieh county in Iran were examined. The results showed that the effect of country and region (districts and villages) on the quality traits of saffron (crocin, picrocrocin and safranal) was significant. Moreover, the comparison of the mean values indicated the presence of significant differences in the qualitative characteristics of saffron among the different investigated villages of Torbat Heydarieh. The saffron produced in Afghanistan had the highest amount of crocin (on average 279.1 ω 440nm) and picrocrocin (on average 101 φ 257 nm), while the highest value of safranal (on average 34.2 θ 330 nm) was observed in saffron samples produced in Iran. The highest amount of the above quality traits belonged to the saffron of Zendeh Jan Herat region 5 (Af_5, crocin: 303 ω 440 nm, picrocrocin: 106 φ 257 nm, safranal: 33 θ 330 nm), while the five Torbat Heydarieh villages had the lowest quality of saffron (Ir_5, crocin: 164 ω 440nm, picrocrocin: 71 φ 257 nm, safranal: 34 θ 330 nm). In the comprehensive analysis of saffron-producing regions in Afghanistan and Iran, in terms of the qualitative attributes (crocin, picrocrocin, and safranal) it was evident that regions 1 to 6 in Herat, Afghanistan (Af1, Af2, Af3, Af4, Af5, Af6) and the Abrood village of Torbat Heidarieh, Iran (Ir7) formed a distinct cluster, demonstrating superior quality compared to other regions. Furthermore, with the exception of the Feizabad village of Torbat Heydarieh (Ir5), all examined samples surpassed the saffron ISO international standard and were categorized as first-grade quality. The exceptional quality of Herat saffron from Afghanistan is likely attributable to the unique geographical features, virgin lands, and specific climatic conditions across diverse cultivation areas.

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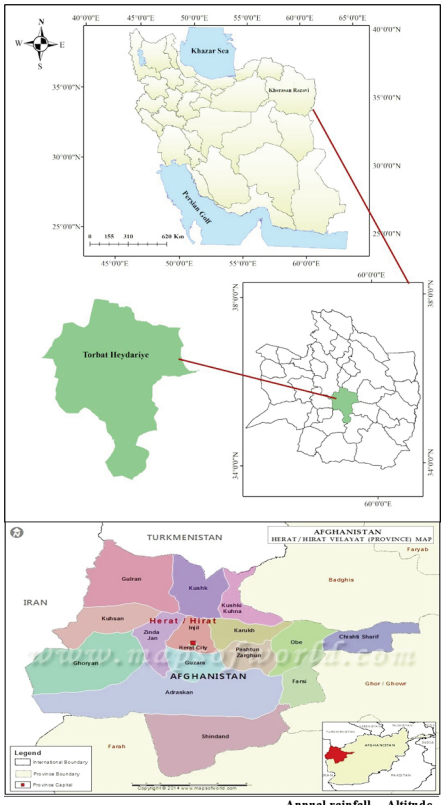
1. Introduction

Saffron (*Crocus sativus* L.) is one of the world's most valuable and expensive medicinal and industrial plants. Originally confined to limited geographic habitats, its significance has led to its widespread cultivation in various parts of the world (Mathew, 1999). In addition to Iran, saffron is also cultivated in other countries such as Afghanistan, India, Spain, Italy, Morocco, Turkey and Greece. In 2019, Afghanistan, as the fourth producer in the world, exported about six tons of saffron (ASYB, 2021), while Iran was the world largest producer with 281 tons of saffron in 2021 (SCI, 2021). Due to the high economic value of saffron, its cultivated area is expanding rapidly in the neighboring countries of Iran, such as Afghanistan and Pakistan (FAO, 2018). Recently, Afghan farmers have developed saffron cultivation in Herat province. Also, the Afghan government is encouraging farmers to grow saffron instead of poppy (*Papaver samniferum* L.) to improve their livelihoods. Herat province is geographically located in the neighborhood of Khorasan province in Iran. In addition Khorasan is the largest saffron production center in the world (Nazarian *et al.,* 2018). Khorasan-Razavi and South Khorasan provinces in Iran are considered the main saffron production pole in the world (Mollafilabi and Khorramdel, 2016). Herat province of Afghanistan is the main center of saffron production in the country, so farmers are engaged in saffron production in almost all the cities of this province (Nazarian *et al.,* 2018). The production method of saffron is slightly different in Iran and Afghanistan. Compliance with the technical principles of cultivation, the use of inputs, and the method of drying saffron are not the same in the two aforementioned countries. So that these factors may have been able to affect the quantity and quality of saffron production.

The stigma of three branches of saffron, which has a red color, contains three main compounds: crocin, picrocrocin and safranal (Tajik *et al.,* 2012). Saffron is widely used in the food and pharmaceutical industries, and due to its therapeutic effects on cancer and depression, it is receiving increasing attention from consumers and processing industries (Lechtenberg *et al.,* 2008). The medicinal and nutritional importance of saffron is related to the existence of a set of secondary metabolites in the stigma. Due to the presence of these metabolites and high amounts of crocin, picrocrocin, and safranal, saffron has the ability to provide color, a special taste and a pleasant aroma in food products. Limited spices have a similar ability to simultaneously provide color, aroma and flavor in the food product to which they are added (Valle Garcia-Rodriguez *et al.,* 2014). Considering the importance of secondary metabolites such as crocin, picrocrocin, and safranal in determining the nutritional and medicinal value of saffron; the measurement of these three parameters can be used as a suitable indicator of quality (Zalacain *et al.,* 2005). To determine the quality of saffron in Iran, in addition to using the ISO international standard (ISO/TS 3632-1 and 3632-2), the national standard (No. 1/259 and 2/259) is also used. Saffron is widely used in the food and pharmaceutical industries. Saffron quality is usually determined by three important secondary metabolites in the stigma i.e. crocin, picrocrocin and safranal, known as effective substances. It has been confirmed that temperature is the main factor controlling the flowering behavior of saffron (Koocheki *et al.,* 2009). In a prior study conducted by Feizi and Moradi (2019), it was revealed that the saffron stigma yield exhibited a positive correlation with regional rainfall, organic matter, nitrogen, phosphorus, and soil sand percentage. Conversely, there was a negative correlation with average temperature and soil clay percentage. The authors also highlighted that altitude above sea level serves as a significant spatial parameter influencing the production of secondary metabolites. Additionally, their findings indicated positive correlations between altitude and picrocrocin, safranal and crocin. Similarly, Kaveh and Salari (2018) reported a positive correlation between crocin and both picrocrocin and safranal. Conversely, both crocin and safranal exhibited a negative correlation with altitude. Moreover, it has been stated that soil pH within the neutral to slightly alkaline range is positively correlated with saffron quality (Gresta *et al.,* 2008). In general, the crop quality is influenced by various factors, exhibiting variation across different regions, such as agricultural practices, nutrition, soil type, water management and air conditions, and ultimately the method employed for saffron drying. On this basis, the purpose of this research was to compare the quality characteristics of saffron produced in Herat province of Afghanistan and Torbat Heydarieh county of Iran.

2. Materials and Methods

In order to compare the qualitative characteristics of saffron in Afghanistan and Iran, samples of dried saffron from seven different districts of Herat province (Injil, Karukh, Guzara, Pashtun Zarghun, Zendeh Jan, Ghoryan, Obeh) of Afghanistan and nine saffron-producing villages (Fakhrabad, Kadkan, Nasar, Ghaleno, Feizabad, Khorgh, Abrood, Benhang, Shadmehr) of Torbat Heydarieh county of Iran (Fig. 1) were collected in the year 2021. The experimental data underwent analysis through the General Linear Model (GLM) procedure. Within the GLM, seven samples from Afghanistan (Af) and nine samples from Iran (Ir) were defined, each with three replications, and the saffron samples were nested over countries. Unlike a factorial model, the nested model differs in that the levels of the second factor (saffron samples) vary within each level of the first factor (country), making it unfeasible to estimate interaction effects in this model. Mean values of qualitative characteristics were compared using the Fisher LSD test. To assess similarities both between and within samples from the two countries, the collected data underwent cluster analysis. All statistical analyses were conducted using Minitab®21 (https://www.gmsl.it/minitab-analisi-dati/).



Districts	Coordinates	Annual rainfall (mm)	Altitude (m)
Ghoryan	34°20′24″N 61°28′12″E	201.0	790
Zenda Jan	34°20′44″N 61°43′12″E	201.0	835
Injil	34°18′00″N 62°15′00″E	238.9	920
Guzara	34°13′0″N 62°13′18″E	238.9	920
Pashtun Zarghun	34°17′0″N 62°37′0″E	241.0	1150
Obeh	34°22′11″N 63°10′35″E	288.0	1277
Karukh	34°29′32″N 62°35′32″E	301.0	1320
City			
Torbat Heydarieh	35°16′26″N 59°13′10″E	247.0	1450

Fig. 1 - Map and geographical characteristics of the studied regions in Herat province of Afghanistan and Torbat Heydarieh county of Iran.

The quality characteristics of the saffron samples were determined by the laboratory of the Saffron Institute of University of Torbat Heydarieh. To determine the amount of crocin, picrocrocin and safranal, the spectrophotometer method (ultraviolet-visible spectrophotometry) and the ISO/TS 3632 standard were used. For this purpose, 500 mg of each saffron sample was ground and after transferring to a 1000 ml volumetric flask, 900 ml of distilled water was added to it. Then it was stirred by a magnetic stirrer for one hour at a speed of 1000 rpm. The volume of the resulting solution was brought up to 1000 ml with distilled water and again placed in the mixer to obtain a uniform solution. The amount of 20 ml of the resulting solution was brought to a volume of 200 ml and stirred until a uniform solution was formed. The solution was filtered away from light, and after obtaining a clear solution, the amount of light absorption in the wavelengths of 200 to 700 nm was recorded compared to the reference (distilled water). The absorption value in the range of 257 nm was related to picrocrocin, in the range of 330 nm to safranal, and in the range of 440 nm to crocin. The reported number was obtained from the following equation (Kaveh and Salari, 2018):

$$E = D \times 1000 / m\ (100 - H)$$

where E is the value of each color compound, D is the absorption value (the number recorded in the spectrophotometer), m is the weight of the saffron sample in grams, and H is the weight percentage of moisture and volatile substances in the sample.

Minitab software was used for statistical analysis, and the means were compared by lest significant difference test (LSD).

3. Results and Discussion

The results of nested analysis of variance showed that the effect of country and regions on quality traits of saffron (picrocrocin, safranal, and crocin)

was significant (Table 1). Moreover, the comparison of mean values indicated the presence of significant differences in the qualitative characteristics of saffron among the investigated regions of Herat, and Torbat Heydarieh (Table 2). The saffron produced in Afghanistan had the highest amount of crocin (with an average of 279.1 ω 440 nm) and picrocrocin (with an average of 101 φ 257 nm), while the highest amount of safranal (with an average of 34.2 θ 330 nm) was observed in the samples of saffron produced in Iran. The highest amount of crocin (303 ω 440 nm) and picrocrocin (106 φ 257 nm) belonged to the saffron sample of Zendeh Jan region and the highest amount of safranal (39 θ 330 nm) was observed in the 6thsaffron sample of Torbat Heydarieh (Ir_6) (Table 2).

In the cluster analysis of qualitative traits of saffron produced in different regions of Herat, four clus-

Table 1 - Analysis of variances (MS) for saffron quality parameters in different regions of Herat Province – Afghanistan and Torbat Heydarieh, Iran

Source of Variation	Df	Picrocrocin (φ 257 nm)	Safranal (θ 330 nm)	Crocin (ω 440 nm)
Country	1	1547.15 **	89.76 **	20539.1 **
Region	14	168. 05 **	21.47 **	2545.5 **
Error	32	0.98	0.95	0.98
Total	47			

** and * are significant at the 0.01 and 0.05 levels of probability, respective.

Table 2 - Mean comparison of quality parameters of saffron from different regions of Herat Province - Afghanistan and Torbat Heydarieh, Iran

Country	Region name	Picrocrocin (φ 257 nm)	Safranal (θ 330 nm)	Crocin (ω 440 nm)
Afghanistan		101 a	31.4 b	279.1 a
Iran		89.6 b	34.2 a	237.4 b
LSD 0.05		0.291	0.285	0.291
Af1	Injil	103 c	30 f	281 d
Af2	Karukh	104 bc	29 f	289 b
Af3	Guzara	101 d	34 cd	270 f
Af4	Pashtun Zarghun	104 bc	29 f	283 c
Af5	Zendeh Jan	106 a	33 de	303 a
Af6	Ghuryan	97 e	29 f	275 e
Af7	Obeh	92 gh	36 b	253 i
Ir1	Fakhrabad	93 fg	30 f	252 i
Ir2	Kadkan	88 k	36 b	236 j
Ir3	Nasar	91 hi	33 de	259 h
Ir4	Ghaleno	90 ij	32 e	236 j
Ir5	Feizabad	71 m	34 cd	164 m
Ir6	Khorgh	89 jk	39 a	227 k
Ir7	Abrood	105 ab	35 bc	289 b
Ir8	Bengang	94 f	34 cd	265 g
Ir9	Shadmehr	85 l	35 bc	209 l
LSD 0.05		0.816	0.799	0.816

Means with the same letters are not significantly different based on the LSD test at 1% probability level P≤0.01.

ters were observed. The highest amount of the above qualitative traits belonged to zone 5 of Zendeh Jan (Af_5, crocin: 303 ω 440 nm, picrocrocin: 106 φ 257 nm, safranal: 33 θ 330 nm), and the lowest amount was belonged to zone 7 of Obeh (Af_7, crocin: 253 ω 440 nm, picrocrocin: 92 φ 257 nm, safranal: 36 θ 330 nm). Also, the above qualitative traits in saffron were obtained from Torbat Heydarieh region of Iran in four different clusters including (Fakhrabad: Ir_1, Nasar: Ir_3, Benhang: Ir_8), (Kadkan: Ir_2, Ghaleno: Ir_4, Khorgh: Ir_6, Shadmehr: Ir_9), Abrood: Ir_7 and Feizabad: Ir_5, so that region 5 had the lowest quality of saffron (Ir_5, crocin: 164 ω 440 nm, picrocrocin:71 φ 257 nm, safranal:34 θ 330 nm). It should be noted that in terms of quality, the different saffron producing regions of Torbat Heydarieh were ranked after the saffron of different regions of Herat. In general, in the examination of the regions of the two countries in terms of the three qualitative attributes of picrocrocin, crocin, and safranal, it was observed that regions 1, 2, 3, 4, 5 and 6 of Herat (Af_1, Af_2, Af_3, Af_4, Af_5, Af_6) and region Abrood of Torbat Heydarieh (Ir_7) was in a cluster and was superior to other regions (Fig. 2).

Also, all the studied samples, except for Torbat Heydarieh Feizabad (Ir_5), were qualitatively higher than the ISO international standard and placed in the first place among first-class saffron (Table 3).

In the review and comparison of the quality of saffron produced in the major production centers of Razavi and South Khorasan provinces, it was reported that out of 14 production centers (Safi Abad, Qaynat, Kashmar, TorbatHydarieh, Khalil Abad, Bardeskan, Shahn-Abad, Chakhmaq, Torbat-Jam, Feiz-Abad, Sabzevar, Sarayan, Taghi-Abad and Birjand) the amount of safranal was lower than the ISO and national standards of Iran in all the investigated villages. For picrocrocin, all regions had values higher than the ISO standard, but regions 2, 10, 11, and 12 could not achieve the minimum national standard, and the product produced in regions 5, 8, and 9 was within the standard limit. Region 12 (Ir_5: Feizabad) had the lowest amount of crocin, which was lower than the ISO standard. Regions 2, 5, 10 and 14 also had crocin values lower than the national standard of Iran (Kaveh and Salari, 2018). The study of the quality characteristics of saffron in Herat-Afghanistan showed that the difference between picrocrocin and safranal was significant in different regions. The highest amount of picrocrocin (104/50 φ 257 nm) and safranal (34/95 θ 330nm) was observed in the saffron

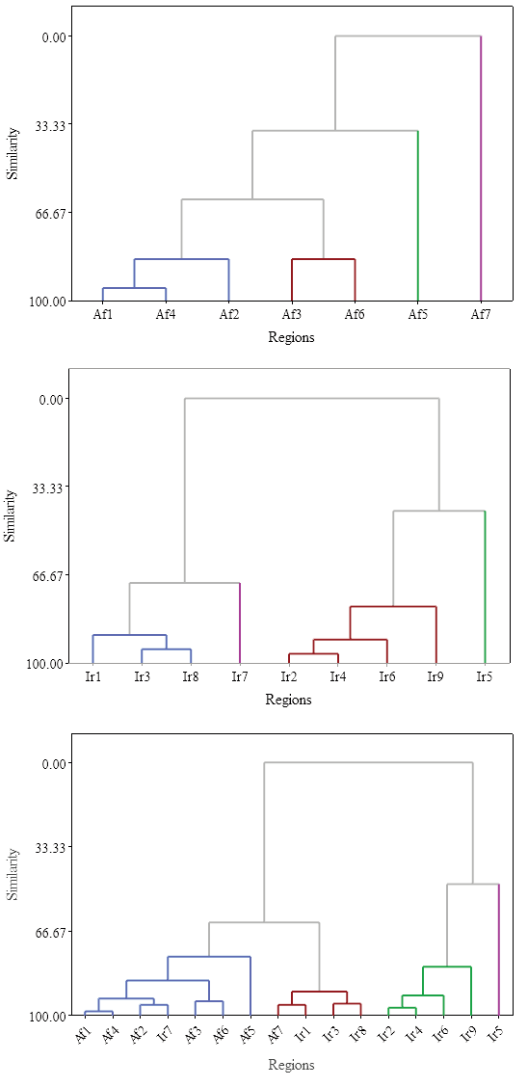


Fig. 2 - Dendogram for hierarchical cluster analysis of 16 studied locations based on crocin, picrocrocin and safranalin Heart, Afghanistan and Torbat Heydarieh, Iran. Ir1- Ir9 (Ir1: Fakhrabad, Ir2: Kadkan, Ir3: Nasar, Ir4: Ghaleno, Ir5: Feizabad, Ir6: Khorgh, Ir7: Abrood, Ir8: Benhang, Ir9: Shadmehr) are 9 saffron producing regions of Torbat Heydarieh, Iran and 7 different district of Heart, Afghanistan are: Af1: Injil, Af2: Karukh, Af3: Guzara, Af4: Pashtun Zarghun, Af5: Zendeh Jan, Af6: Ghuryan, Af7: Obeh.

Table 3 - Sample classification based on ISO 3632/1-2 Normative (ISO, 2003)

ISO category	E 1% 257 nm	E 1% 440 nm
I Grade	70	190
Grade II	55	150
Grade III	40	100

Accordingly, to the absorbance readings at different wavelengths of solutions of the same concentration E1% ($w.v^{-1}$) at 257 and 440 nm. Source: Gresta *et al.*, 2008.

samples of the saffron-producing regions, while the highest amount of crocin (236/95 ω 440nm) was recorded in the saffron samples of Pashtun Zarghun city. Also, by comparing the studied samples with the international saffron standard (ISO 3632), it was observed that all the samples were higher than the group grade 1 (Nazarian *et al.,* 2021).

In the world, there are many differences in product quality parameters from one region to another. The reported values for crocin range from 0.85 to 32.4 percent of dry matter (Alonso *et al.,* 1999), safranal from 0.026 to 0.29 mg/dry weight (Hadizadeh *et al.,* 2006), picrocrocin 2.18 to 6.15 percent of dry matter (Straubinger *et al.,* 1998) has been reported for Iranian saffron. The results showed that there is a positive correlation between picrocrocin with safranal, crocin and altitude above sea level (Kaveh and Salari, 2018). The correlation of traits with altitude was consistent with the results of (Lage and Cantrell, 2009). Studies conducted on the correlation of saffron quality and geographical conditions of various factors such as soil, altitude above sea level and temperature have been reported as factors with positive or negative correlation. In a study on the qualitative characteristics of saffron in Morocco, it was reported that the quality of the soil had no effect on the quality of saffron (Lage and Cantrell, 2009). In another study, it has been stated that soil acidity in the range of neutral to slightly alkaline has a positive correlation with saffron quality (Gresta *et al.,* 2008). In many countries, researchers reported that the chemical composition of saffron samples can be strongly influenced by weather conditions, agricultural management practices, harvesting methods, and methods used to dry saffron stigma (Lozano *et al.,* 2000; Zareena *et al.,* 2001; Kanakis *et al.,* 2004; Behdani and Fallahi, 2015). Based on the above reports, it can be said that the amount of saffron compounds in different countries may be very different. During the last two decades, saffron cultivation in Afghanistan has developed significantly, and it has been promoted in most provinces of the country. Afghan farmers do not pay enough attention to saffron nutrition management due to the bad economic situation. Therefore, they use the least amount of animal manure and chemical fertilizers in saffron cultivation. The saffron samples examined in this research were selected from different regions of Herat province with different geographical characteristics (altitude, amount of rainfall, latitude, etc.). The results showed that these different geographical characteristics can affect the quality of saffron produced. Zendeh Jan region with an altitude of 835 m and an annual rainfall of 201 mm was produced the best quality of saffron, while the quality of saffron decreased with the increase of the altitude and the amount of rainfall, so that Obeh region in Herat and samples of saffron produced in Torbat Heydarieh were have the lowest quality of saffron. On the other hand, due to the simultaneous growth of saffron with winter rainfall, the water required by saffron can be supplied by irrigating 2 to 3 times in this way. The method of drying saffron, which affects the quality of saffron, is completely traditional in Afghanistan, and without the use of machines. Therefore, according to the prosperity and development of saffron cultivation in different regions and the virgin lands of Herat province, which have not been under the cultivation of this new and economic product, the qualitative superiority of saffron produced in different regions of Herat compared to the samples of Torbat Heydarieh county, for the amount of secondary metabolites such as crocin, picrocrocin and safranal does not seem unlikely.

4. Conclusions

The chemical composition of saffron stigma can be strongly influenced by weather conditions, altitude, farm management practices, harvesting methods, and the approach to stigma drying. In general, the samples from regions 1 to 6 (Anjil, Karukh, Guzarah, Pashtun Zarghun, Zendeh Jan and Ghoryan) of Herat province, Afghanistan and Abrood region (Ir_7) of Torbat Heydarieh, Iran, exhibited higher levels of crocin and picrocrocin compared to the ISO standard.These samples not only secured the first-place ranking but also surpassed saffron samples from various regions in Torbat Heydarieh, Iran. This superiority can be attributed to the unique geographical features, the virgin nature of the land dedicated to saffron cultivation for the first time, and the different climatic conditions in the cultivated regions.

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Review paper

AHS
Advances in Horticultural Science

Efficacy of active and passive modified atmosphere packaging on quality preservation and storage life of pomegranate fruit and arils: A review

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Abstract: Pomegranate has nutritional value and health benefits due to its bioactive compounds and antioxidant properties. Fruit consumption is strongly recommended due to its high content of vitamins, fiber, minerals, and polyphenols. Supplying ready-to-eat pomegranate can be a beneficial technique to increase consumption with regard to its nutritional properties. However, maintaining nutritional quality and preventing microbial spoilage is a major challenge. Fruit quality is lost with visible symptoms such as weight loss, shriveling, husk scald, chilling injury, fungal rot, aril color degradation, and off-flavor during long-term storage. Therefore, it is very important to use appropriate strategies to maintain pomegranate whole fruit and aril quality. Gases around the product create a suitable environment for oxidative reactions and aerobic microorganism growth. Therefore, changing the atmosphere around the product can help maintain its quality. One of the effective methods to increase the postharvest life of products is to use modified atmosphere packaging (MAP), which reduces microbial spoilage and chilling injury, preserves the quality, and extends the shelf life by reducing the respiration rate. Modified atmosphere packaging, which uses natural atmospheric components (O_2, CO_2 and N_2), has been widely accepted due to the lack of toxic residues on the product. This review discusses on recent research in terms of MAP application on quality properties and postharvest life of pomegranate fruit and arils during storage.

1. Introduction

Regarding botanical classification, pomegranate belongs to the *Angiospermae* category, *Dicotyledoneae* subcategory, *Myrtales* order, *Lythraceae* family, *Punica* genus, and *P. granatum* species. *P. granatum* species is diploid (16x=2 n=2). It has four subspecies: *plenty-flora*, *spinisia*, *nana*, and *sativa*. Edible pomegranate is in the subspecies of *sativa*

(Jalikop, 2010). This fruit is mainly grown in Spain, Turkey, Egypt, Tanzania, Saudi Arabia, Azerbaijan, Pakistan, Afghanistan, India, and China. Among these countries, India, Iran, China, and Turkey are the main pomegranate producers (Ramezanian and Erkan, 2017). Pomegranate has many nutritional properties and bioactive compounds with anti-inflammatory, antioxidant, anticancer, antihypertensive, antidiabetic and liver damage-reducing effects (Kalaycıoglu and Erim, 2017; Khajebishak *et al*., 2019; Sohrab*et al*., 2019; Barati Boldaji *et al*., 2020; Firdous *et al*., 2023). The pomegranate is considered a non-climacteric fruit, and is harvested at the optimal maturity stage for storage, which has optimal organoleptic characteristics. The harvest index is the ratio of sugar to acid, and the standard index for harvesting is different depending on the cultivar (Artés *et al*., 2000). Post-harvest quality loss due to weight loss, hardening of the husk, cracking husk, chilling injury (CI) symptoms and fungal diseases limit its storage potential (Pareek *et al*., 2015; Porat *et al*., 2016; Ranjbari *et al*., 2018; Candir *et al*., 2019; Lufu *et al*., 2020) (Fig. 1). Moreover, ready-to-eat pomegranate aril is very perishable and rapidly lost its quality during storage. The most important goal of the postharvest industry is to maintain the quality during transportation and storage (El-Ramady *et al*., 2015; Moradinezhad and Dorostkar, 2021). Reducing postharvest losses leads to more available food, reducing cultivated areas, and preserving natural resources. Therefore, it is necessary to use techniques to maintain the fruit quality after harvest and during storage. Changing the atmosphere around the fruit through controlled atmosphere (CA) or modified atmosphere packaging (MAP) is a reliable and safe approach (Caleb *et al*., 2012; Caleb *et al*., 2013 a, b). In MAP, the gas composition inside the package is obtained based on the gas exchange through the semi-permeable layer and fruit respiration rate (Caleb *et al*., 2018). Although the respiration rate of pomegranate fruit is slow in cold temperatures, however, during the respiration process, oxygen (O_2) is consumed and dioxide carbon(CO_2) is produced, which changes the composition of the gas inside the package (passive MAP). In addition to passive MAP, the initial modification of respiratory gases (active

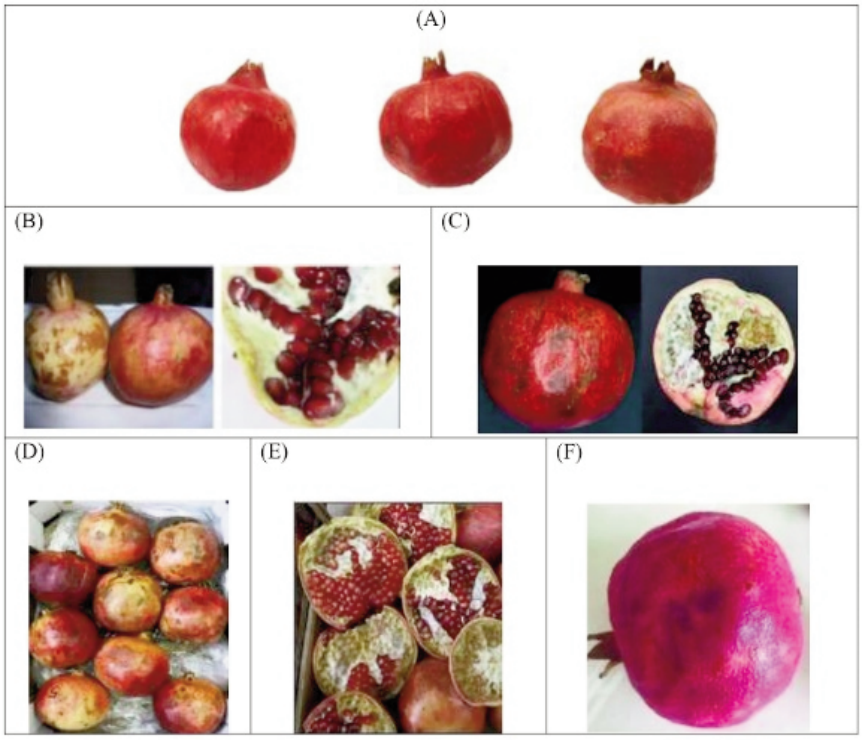

Fig. 1 - Chilling injury development in pomegranate cultivars stored at cold storage. A) The external appearance and weight loss of 'Wonderful'pomegranate fruit after 4, 8, and 12 weeks of storage at 7°C, photo caption from left to right, respectively (Adetoro *et al*., 2020), B) The external and internal appearance of 'Mengzi Sweet' pomegranate fruit after 8 weeks of storage at 2°C (Chen *et al.*, 2021), C) Husk scald and chilling injury in pomegranate 'Wonderful' fruit skin after 120 days of storage at 3.5°C (Maghoumi *et al.*, 2022), D) Husk scald symptoms of 'Wonderful' pomegranate fruit after 12 weeks of storage at 7°C (Li *et al*., 2016), E) The internal appearance and discoloration of the texture of 'Wonderful' pomegranate fruit after two weeks of storage at 1°C (Kashash *et al*., 2019), F) Hard husk and shriveling symptoms of 'Hicaznar'pomegranate fruit after 6 months of storage at 6°C (Candir *et al.*, 2019).

MAP) based on the physiology of the product, environmental conditions and the properties of the packaging materials has a significant effect in reducing respiratory activity and increasing the shelf life (Opara *et al*., 2015; Opara *et al*., 2017; Belay *et al*., 2018; Dorostkar and Moradinezhad, 2022).

Despite the advantages of MAP, ultra-low or high concentrations of gases inside packages may cause damage to the texture. An excessive increase in O_2 concentration increases the production of radicals that damage the cytoplasm, such as superoxide (O^{-2}), hydrogen peroxide (H_2O_2), and hydroxyl (OH^-), consequently reducing the quality by inhibiting some metabolic activities (Choudhury *et al*., 2017). The reduction of O_2 below the critical limit causes the initiation of anaerobic respiration and fermentation, resulting in an unpleasant aroma and taste (Li *et al*., 2014). Also, excessive accumulation of CO_2 can lead to a decrease in quality by accelerating color changes and increasing the hydrolysis of pectin compounds (Teixeira *et al*., 2016).

With the increasing demand for MAP application, it is necessary to understand the role of gases and their effect mechanism on product quality. Therefore, a simplex lattice design approach was considered to select and identify the optimal gas composition to maximize the quality parameters of pomegranate aril cv. Wonderful under modified atmosphere conditions (Belay *et al*., 2019 a). The partial pressure of gases as visual quality, physicochemical characteristics, antioxidant properties and volatile organic compounds (VOCs) were selected as response variables. The results showed that CO_2 was the most important factor affecting color, texture firmness and volatile organic compounds (aldehydes, ketones, monoterpenes) of the Wonderful cultivar (Li *et al*., 2018; Li *et al*., 2020). O_2 had the greatest effect on color, organic acid, decay development and alcoholic volatile organic compounds. The maximum concentration of sugars, organic acids, total soluble solids (TSS), and color using a gas mixture (6-7 kPa O_2+ 7-8 kPa CO_2),and the maximum release of volatile compounds responsible for the taste of arils was obtained using a gas mixture (2 kPa O_2 + 18 kPa CO_2 + 80 kPa N_2) (Belay *et al*., 2019 a).

Low O_2 inhibits the rate of oxidation by reducing the rate of respiration and delaying fruit ripening (Li and Zhang, 2015; Teixeira *et al*., 2016). The concentration of super atmospheric $O_2$was effective in inhibiting microbial growth and reducing decay by preventing anaerobic respiration on minimally processed pomegranate arils (cv. Wonderful) (Belay *et al*., 2017 b). The qualitative changes of the fruit are related to the change of different metabolic pathways which are presented in the modified atmosphere by determining the genomic interpretation and their transcription frequency under the influence of packaging conditions (Rosales *et al*., 2016). The response of fruits to gas concentration is characterized by the profile of primary metabolite (respiration rate) and secondary metabolite (fermentative metabolites and volatile compounds) (Blanch *et al*., 2015).

Despite the advantages of the modified atmosphere in increasing the shelf life of the product, reducing storage losses without preservatives application, accurate control of storage temperature due to the effect of temperature on the permeability of used films, respiration rate, and solubility of gas in the aqueous phase of the food and the nutrient leakage, and determination of the specific gas composition for each product should be investigated.

Considering the importance of the storage environment, especially the concentration of O_2 and CO_2 in the occurrence of injury symptoms and the shelf life of products, this review aimed to investigate the efficacy of MAP on the overall quality of the whole pomegranate fruit and arils during cold storage.

2. Influence of MAP on quality traits of pomegranate

Chilling injury, weight loss, and overall quality

One desirable approach to minimize weight loss in a modified atmosphere is to reduce respiratory activity, which substantially reduces transpiration (Belay *et al*., 2018). Therefore, in the MAP, it is recommended to choose the appropriate gas composition to control the weight loss of the product. In the investigation of the suitable gas composition to reduce the respiration rate, the concentration of O_2 (2, 10 and 21 kPa) and CO_2 (2, 10 and 20 kPa) on pomegranate arils cv. Hicaznar (Ersan *et al*., 2009), and the concentration O_2 (5, 21, and 30 kPa) and CO_2 (0, 10, and 40 kPa) on the pomegranate arils cv. Wonderful (Banda *et al*., 2015) stored at 5°C showed that low O_2 concentration significantly decreases the respiration rate. Also, the concentration of O_2 2-4 kPa is recommended to maintain the quality of pomegranate arils cv. Mollar de Elche (López-Rubira *et al*., 2005). In a study on pomegranate arils cv. Wonderful in modi-

fied atmosphere (4.67kPa O_2, and 12.67kPa CO_2) packed with PropaFilm and Nature Flex showed that arils packed in PropaFilm had lower mass loss than NatureFlex, due to the film's lower water vapor transmission rate (WVTR) (Belay *et al*., 2018). High gas barrier properties PropaFilm, even at high relative humidity, lead to the potential for shelf-life extension. Weight changes are related to changes in respiration and transpiration, which are influenced by the difference in diffusion resistance and the surface to volume ratio of pomegranate arils (Khorshidi *et al*., 2011). Long-term storage of pomegranate arils causes more weight loss due to higher enzyme activity and lower resistance of the cell membrane against water loss (Belay *et al*., 2018). Modified atmosphere packaging reduces the vapor pressure difference between the surface and environment of the product by maintaining the relative humidity around the fruit, accordingly reducing the water loss of the product (Ngcobo *et al*., 2013).

Water loss of whole pomegranate fruit causes husk browning at the storage (Nerya *et al*., 2006). Also, enzymatic browning after microbial infection is the main cause of quality reduction (Ioannou and Ghoul, 2013) that polyphenol oxidase (PPO) and peroxidase (POD) activity increases the brown superficial discoloration of pomegranate fruits (Xie *et al*., 2019; Baghel *et al*., 2021). Storage of pomegranates cv. Mollar de Elche in controlled atmospheres (10kPa O_2 and 5kPa CO_2; 5kPa O_2 and 5kPa CO_2; 5kPa O_2 and 10kPa CO_2;or 5kPa O_2 and 0kPa CO_2) for 8 weeks at 5°C showed that all treatments except 10kPa O_2 and 5kPa CO_2 reduced weight loss, fungal rot and chilling injury symptoms (husk scald) and were efficient for increasing the quality and extending the shelf life of pomegranate fruits (Artés *et al.*, 2000). Moreover, storage of pomegranate fruit in a controlled atmosphere (1 kPa O_2 +15 kPa CO_2 or 5 kPa O_2 +15 kPa CO_2) significantly reduced botrytis rot and scald for up to 6 months at 7°C (Defilippi *et al*., 2006; Palou *et al*., 2007). Pomegranate fruits stored in a modified atmosphere (5kPa CO_2 + 3kPa O_2) for three months at 5°C had wrinkle-free husk and smoother, less chilling injury, fewer disease symptoms and, as a result, better quality compared to fruits stored in a normal atmosphere (Sidhu *et al*., 2019). Pomegranate fruits stored in a modified atmosphere (5-10 kPa CO_2 + 3-5 kPa O_2) increases shelf life due to reduced weight loss, decay and injury symptoms (Selçuk and Erkan, 2015; Porat *et al*., 2016; Maghoumi *et al*., 2022). High CO_2 concentration is effective in maintaining the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) (Song *et al*., 2013), and on the other hand, due to the lower O_2 concentration, POD and PPO enzymes do not catalyse the oxidation of phenols (Ali *et al*., 2019). Also, increasing the concentration of CO_2 and decreasing the concentration of O_2 will inhibit fungal contamination by suppressing respiration (Almenar *et al*., 2006). As mentioned, water stress, oxidative stress, lipid peroxidation and cell membrane instability are key factors in burn development (Singh *et al*., 2018). MAP and CA prevent husk scald by limiting oxygen access, oxidative stress and water loss prevention. It has been proved that MAP is effective in maintaining the external and internal quality of pomegranate fruit by controlling weight loss, and preventing fungal decay and husk scald during cold storage (Selçuk and Erkan, 2015; Porat *et al*., 2016).

Fruit weight loss increases CI symptoms by destroying the membrane integrity (Opara *et al*., 2015; Maghoumi *et al*., 2023). The decrease in unsaturated fatty acid content and membrane fluidity causes damage to the membrane structures and a lack of resistance to cold (Casares *et al*., 2019). It has been reported that CI symptoms coincide with the leakage of electrolytes in the pomegranate peel (Casares *et al*., 2019). Oxidative damage, membrane chilling injury and electrolyte leakage in pomegranate peel are indicated as a function of O_2 levels in the first days of storage (Valdenegro *et al*., 2018). In the modified atmosphere condition, the stability of SOD and CAT enzymes leads to less accumulation of H_2O_2 and malondialdehyde (MDA), more integrity of the membrane and therefore less electrolyte leakage (Li *et al*., 2016; Valdenegro *et al*., 2022), and a higher PAL/PPO ratio reduces oxidative damage (Baghel *et al*., 2021). Researchers have studied extensively the effect of a low-oxygen atmosphere on the quality characteristics of whole pomegranate fruit or arils cv. Primosole (D'Aquino *et al*., 2010), cv. Acco and Herskawitz (Caleb *et al*., 2013 a, b), cv. Wonderful (Banda *et al.,* 2015), cv. Hicaznar (Candir *et al*., 2018), cv. Shishe-Kab (Moradinezhad *et al*., 2013, 2019), and it has been found that the atmosphere with low oxygen has the potential to prevent weight loss, chilling injury, decay and delay in post-harvest ripening (Table 1).

Firmness

The firmness reduction is related to water loss,

cell membrane deterioration and senescence (Díaz-Mula *et al*., 2012; Hussein *et al*., 2015). The effect of MAP on maintaining fruit firmness is related to the control of weight loss, which has an important effect on postharvest management (Jouki and Khazaei, 2014). Also, maintaining post-harvest firmness is related to the control of biochemical processes (activities of pectinsterase and polygalacturonase enzymes) (Fagundes *et al*., 2015; Bang *et al*., 2019) and the prevention of ethylene synthesis under a modified atmosphere (Akbudak *et al*., 2012). High CO_2 inhibits ethylene production and delays ripening (Kader and Watkins, 2000). A similar effect of high CO_2 and super atmospheric O_2 has been reported on firmness of aril cv. Wonderful that arils stored under super atmospheric O_2 (70%) showed a slight increase in the firmness compared to low O_2 treatment (5 and 10%) (Belay *et al*., 2017 b). A low respiration rate limits the activity of cell wall-degrading enzymes (such as pectinase and cellulase) and preserves firmness during storage (Fagundes *et al*., 2015; Bessemans *et al*., 2016), and as a result delayed ripening (Mahajan *et al*., 2014; EL-Eryan*et al*., 2020). MAP can lead to structure preservation, less tissue damage and shelf-life quality of aril due to increased vapor pressure and reduced cell wall polysaccharides degradation (Zhao *et al*., 2019).

Color characteristics

Fruit color is related to the breakdown of chloroplasts, chromoplasts and the change of natural pigments (chlorophylls, anthocyanins, carotenoids, flavonoids) that are affected by packaging and storage conditions (Yin *et al*., 2016). L^*, a^*, b^* values represent the lightness, redness and yellowness. Chroma (C^*) and hue angle ($h°$) describe the color intensity and purity, respectively. L^*, C^* and $h°$ indices reflect the intensity of the color. A slight decrease in C^* and an increase in $h°$ indicates the loss of color intensity of pomegranate arils during storage (Palma *et al*., 2015). Loss of the color intensity during MAP can be controlled by regulating enzymatic and non-enzymatic activities through decreasing O_2 concentration or reducing water loss (Belay *et al*., 2018). High CO_2 concentration prevents enzymatic browning by reducing phenolic substrate and PPO activity (Manolopoulou and Varzakas, 2013). Belay *et al*. (2017 b) reported that MAP, storage time and their interaction had a significant effect on color intensity of pomegranates cv. Wonderful stored at 5°C. The highest C^* was observed under a low O_2 atmosphere (5kPa), while the super O_2 atmosphere (70kPa) maintained initial C^* values during storage (Belay *et al*., 2017 b).

Titratable acidity (TA) and total soluble solids (TSS)

The reduction in TA of pomegranate juice cv. Mollar de Elche without changes in TSS was observed under UV-C treatment and super atmospheric O_2 conditions, which is related to metabolic activities and increased catabolism of organic acids in the respiration process (Maghoumi *et al*., 2013). On the other hand, increasing TA of arils cv. Kingdom and MR-100 under the passive modified atmosphere at 5°C were due to fermentation, which was confirmed by the growth of total aerobic bacteria, yeasts, and molds (Adiletta *et al*., 2017). Changes in gas composition (increase and/or decrease O_2 or CO_2) hydrolyze polysaccharides to sugars (Sucrose, glucose, and fructose) by changing the activity of carbohydrate biosynthesis enzymes and sugar compound metabolism. The active modified atmosphere provides high non-reducing sugars at the end of storage, which can affect the chemical, sensory and quality characteristics of pomegranate arils during storage (Patanè *et al*., 2019; Moradinezhad *et al*., 2020). In addition, an increase in sugar content (fructose, glucose, and sucrose) of pomegranate cv. Wonderful was observed in 4.6kPa O_2 and 12.65kPa CO_2 (Belay *et al*., 2018), likely because that exposure to CO_2 preserves energy reserves. The reduction of TSS in the super-atmospheric is due to the reduction of carbohydrates, pectin, partial hydrolysis of protein and breakdown of glycosides into constituent units during respiration (Blanch *et al*., 2015). The effect of MAP on organic acids and sugars of pomegranate fruit reported as inconsistent and were mainly depended on the cultivar and also the duration of storage, as the TSS value on cv. Mridula increased (Barman *et al*., 2011), while on cv. Ruby decreased (Fawole and Opara, 2013), and on cv. Mollar remained unchanged (Sayyari *et al*., 2011). Therefore, the control of respiration rate (RR) and transpiration rate (TR) is crucial to preserve TA and TSS values during storage as much as possible, in order to get a higher TSS to TA ratio index.

Ascorbic acid, antioxidant and anthocyanin content

The reduction of ascorbic acid (AA) was observed in arils cv. Malese Saveh stored under super atmospheric O_2 (70kPa) for 14 days at 4°C (Maghoumi *et al*., 2014). In investigating the effect of the modified atmosphere, low O_2 (5 or 10kPa O_2, 10kPa CO_2), super

atmosphere (70kPa O_2, 10kPa CO_2) and normal atmosphere on the AA content of pomegranate cv. Wonderful at 5°C, it was found that oxidation AA was associated with the presence of O_2. As a result, the content of AA decreased in the super atmosphere and normal atmosphere (Belay *et al*., 2017 b). However, super atmospheric O_2 has beneficial effects on other quality characteristics. Excessive amounts of O_2 and CO_2 may cause the oxidation of AA through increasing oxidative stress on plant tissues (Belay *et al*., 2017 b). Besides the atmosphere, the nature of the fruit also affects the concentration of AA during storage, so acidity levels are one of the factors affecting the stability of AA during storage (Wahyuningsih *et al*., 2017). The reduction of AA as an antioxidant agent is due to its use as an electron donor to oxidants for neutralizing free radicals is attributed to fruit respiration and sensitivity to chilling injury (Artés *et al*., 2006).

The effect of packaging with different gas compositions (5 kPa O_2 + 10 kPa CO_2 + 85 kPa N_2; 10 kPa O_2 + 5 kPa CO_2 + 85 kPa N_2; 70 kPa O_2 + 10 kPa CO_2 + 20 kPa N_2; 21 kPa O_2 + 0.03 kPa CO_2 + 78 kPa N_2) was investigated on the physicochemical characteristics, nutrient and volatile organic compounds of aril cv. Wonderful for 12 days at 5°C. It was observed that arils packed with low O_2(5 kPa O_2 + 10 kPa CO_2 + 85 kPa N_2) have more nutrients content (Belay *et al*., 2017 b). Higher values of AA, anthocyanin and phenolic compounds were observed in pomegranate cv. Wonderful stored in low O_2 concentration (5 kPa O_2+ 10 kPa CO_2 + 85 kPa N_2 and 10 kPa O_2 + 5 kPa CO_2 + 85 kPa N_2) at 5°C. Also, maintaining low O_2 concentration using low permeability polypropylene film preserved pomegranate anthocyanin and improved sensory quality (Banda *et al*., 2015). Decreasing the respiration rate reduces the amount of carbohydrates, and the carbohydrates that accumulate in the tissue are used in the production of phenolic compounds (Wang *et al*., 2017).

Increasing the activity of antioxidant enzymes, such as SOD, CAT, and APX removes oxygen free radicals and reduces the activity of PPO and POD enzymes involved in the browning of arils cv. Purple Queen was packed in semipermeable film, which had higher polyphenol and anthocyanin content (Adiletta *et al*., 2019). Also, heat treatment, UV-C and super atmospheric O_2 packaging delayed the PPO and glutathione peroxidase (GPX) activity of pomegranate arils cv. Malese-Saveh and maintained the antioxidant concentration (Maghoumi *et al*., 2013). Accumulation of phenolic compounds exposed to high O_2 can be a physiological stress response, and stimulates phenylalanine ammonia-lyase (PAL) activity during minimal processing (Baenas *et al*., 2014). The increase of O_2 in the first days of storage may increase the antioxidant activity, but in the long-term, it reduces the main antioxidants including anthocyanins and phenolic compounds due to the oxidation stimulated by O_2 (Maghoumi *et al*., 2014). Increasing reactive oxygen species (ROS) causes the oxidation of phenolic compounds due to the increase in PPO activity and loss of membrane compartmentalization (Cisneros-Zevallos *et al*., 2014). At the end of the storage of pomegranate arils cv. Wonderful the lowest anthocyanin concentration was observed n high O_2 atmospheres (30kPa O_2 and 10kPa CO_2) (Banda *et al*., 2015), which could be due to the oxidation of AA (Maghoumi *et al*., 2014). Palma *et al*. (2015) related the changes in anthocyanin content to the presence of organic acids (e.g. ascorbic acid) and titratable acidity, which provide the carbon skeleton for the synthesis of secondary metabolites (e g. anthocyanins) during storage (Palma *et al*., 2015). Changes in anthocyanin content can be attributed to the interaction of arils with gas composition, biosynthesis and stability of individual anthocyanins (Palma *et al*., 2015; Moradinezhad *et al*., 2020). Due to the inhibition of anthocyanin biosynthesis in high CO_2, the anthocyanins of pomegranate cv. Wonderful stored in atmospheres enriched with CO_2 (10-20 kPa) were lower compared to fruit stored in air (Holcroft *et al*., 1998). Higher levels of CO_2 in X5 and X12 packages probably delayed anthocyanin synthesis and reduced the intensity of aril color during storage by reducing anthocyanin and phenol (Selcuk and Erkan, 2015; Tzoumaki *et al*., 2009). The reduction in anthocyanin content, which affects the color of arils, is a disadvantage of storage with high CO_2 levels (Table 1).

Volatile organic compounds (VOCs)

The identified VOCs comprised five compound groups (aldehyde, ketone, alcohol, ester and monoterpene), ester compounds were dominant, followed by ketones and aldehydes, whereas, alcohol and monoterpenes were the least abundant (Belay *et al*., 2018). Increasing VOCs are related to the acceleration of metabolism in response to the atmosphere, which can lead to stress and disruption of enzyme systems (Giuggioli *et al*., 2015). Increased VOCs at low O_2 stimulate the production of fermentative

Table 1 a - Efficacy of modified atmosphere packaging (MAP) on whole pomegranate fruit and arils

Pomegranate cultivar	Treatment	Whole fruit/Aril	Storage time (days)	Outcomes	Reference
Hicaznar	Passive modified atmosphere using Xtend® and ZOEpac	Whole fruit	210	Increase of polyphenols, anthocyanins, antioxidant activity, delay in color change and maintain appearance quality up to day 120, maintain physiological and biochemical properties up to day 180.	(Selcuk and Erkan, 2015)
Hicaznar	Passive modified atmosphere using Xtend®	Whole fruit	180	Maintaining husk color, titratable acidity, and ascorbic acid content, and reducing weight loss and husk scald.	(Candir *et al*., 2018)
Shishe-kab	Pre-treatment with short-term high CO_2 and packaging in polyethylene bags, Nano-bags and Decoo Magic Bag	Whole fruit	90	Reducing respiration rate, weight loss, decay, and chilling injury, and maintaining organoleptic properties	(Moradinezhad *et al*., 2018)
Afganski, Crab, Cranberry, Entek-habi-saveh	Modified atmosphere packaging (5 kPa CO_2 + 3 kPa O_2)	Whole fruit	90	The fruit had a wrinkle-free skin, less chilling injury, less disease symptoms and better quality	(Sidhu *et al*., 2019)
Succary	Passive modified atmosphere using high ethylene absorption (HEA), perforated polyethylene (PPE), polyethylene (PE) film, stretchable cling film, poly vinyl	Whole fruit	90	The fruit had a less chilling injury, lower changes in acidity and soluble solid content, and increased antioxidant activity.	(Serry, 2019)
Wonderful	Passive modified atmosphere using non-perforated 'Decco' and 'Zoe', micro-perforated Xtend®, micro and macro perforated high density polyethylene (HDPE)	Whole fruit	84	Packaging whole fruit with micro- and macro- perforation reduced post-harvest losses by minimizing moisture condensation, fruit rot and shriveling.	(Lufu *et al*., 2021)
Wonderful	Passive modified atmosphere using micro-perforated Xtend® and macro-perforated high-density polyethylene	Whole fruit	42	Fruits packaged in the micro-perforated Xtend® had least weight loss, lowest respiration rates, highest total soluble solids and no fungal decay.	(Kawhena *et al*., 2022)
Wonderful	Passive modified atmosphere using XTend™ bags	Whole fruit	120	Increasing the concentration of anthocyanin in the husk and arils, delaying the symptoms of chilling injury up to 120 days	(Valdenegro *et al*., 2022)
Wonderful	Passive modified atmosphere using 100% cellulose-based film NatureFlex (NF), bi-axial-oriented polypropylene (BOPP)-based film PropaFilm (PF), NF-PF (66:33%) film, and PF-NF (33:66%) film	Aril	9	Pakage NF-PF (66:33%) film, and PF-NF (33:66%) film resulted in lowest in-package water vapour condensation and mold growth, and maintained the quality of arils at storage.	(Belay *et al*., 2018)
Purple Queen	Passive modified atmosphere using micro-perforated (MPP) and semipermeable (SP) films	Aril	16	Arils packaged in the SP system had high polyphenols, anthocyanins contents, antioxidant activity (superoxide dismutase, catalase, and ascorbate peroxidase) and low polyphenol oxidase and peroxidase activity.	(Adiletta *et al*., 2019)
Wonderful	Passive modified atmosphere using Xtend bag, polyethylene bag, polypropylene bag, and silver nano bag	Aril	18	Silver nano bag maintained the taste, aroma and overall acceptability, anthocyanin, vitamin C and antioxidant activity and reduced pectinase activity.	(EL-Eryan, 2020)
Wonderful	The nitrogen and argon-based MAP treatment (MAP Ar)	Aril	16	Arils packaged in the (MAP Ar) had high sugar/acid ratio, and desired sensory quali-	(Tinebra *et al*., 2021)

Table 1 b -Efficacy of modified atmosphere packaging (MAP) on whole pomegranate fruit and arils

Pomegranate cultivar	Treatment	Whole fruit/Aril	Storage time days	Outcomes	Reference
Bhagwa	Passive modified atmosphere using transparent high-density	Aril	5	Increasing titratable acidity, anthocyanins reducing sugars, and total soluble solids,	(Rokalla *et al*., 2022)
Rabbab	Passive modified atmosphere using Polyethylene+ Polyester (PE+PES) and Biaxial oriented polypropylene (BOPP) film	Aril	15	PE+PES film caused delay in decreasing the trend of total antioxidant activity and had the lowest number of aerobic mesophilic bacteria and psychrophilic bacteria.	(Ranjbar and Ramezanian, 2022)
Wonderful	Active modified atmosphere based on high O_2	Aril	12	The gas mixture containing 30 kPa O_2 + 10 kPa CO_2 + 60 kPa N_2 had lower aerobic mesophilic bacteria counts, higher sensory scores and long-term shelf life.	(Banda *et al*., 2015)
Wonderful	Active modified atmosphere based on low O_2 and enriched CO_2	Aril	9	The gas mixture containing 12.67–18 kPa CO_2, 2–4.67 kPa O_2 and 80 – 82.67 kPa N_2 reduced microbial count.	(Belay *et al*., 2017 a)
Wonderful	Active modified atmosphere based on low O_2 and super-atmospheric O_2	Aril	12	The gas mixture containing 5 kPa O_2 + 10 kPa CO_2 + 85 kPa N_2 and 10 kPa O_2 + 5 kPa CO_2 + 85 kPa N_2 maintained phytonutrient content, 70 kPa O_2 + 10 kPa CO_2 + 85 kPa N_2 had low aerobic mesophilic bacteria, yeast and mold counts.	(Belay *et al*., 2017 b)
cv. Wonderful	Active modified atmosphere based on low O_2	Aril	9	The gas mixture containing 2 kPa O_2 +18 kPa CO_2 + 80 kPa N_2 leads to the accumulation of ethanol, increase in respiration quotient and oxidation of organic acids.	(Belay *et al*., 2019 b)

compounds (Zhang *et al*., 2013 a; Cortellino *et al*., 2015) and induce cell damage and senescence by producing anaerobic metabolism (Li and Zhang, 2015). Super atmospheric O_2 affects the synthesis and accumulation of some VOCs related to respiratory metabolism (such as acetaldehyde, ethanol, and ethyl esters). Accumulation of acetaldehyde is the first indicator of fermentation metabolism, which is rapidly converted to ethanol by the enzyme alcohol dehydrogenase (ADH) and negatively effects on sensory properties (Thewes *et al*., 2015; Manolopoulou and Varzakas, 2013). The highest amount of VOCs was observed in arils stored under super atmospheric O_2 and enriched CO_2 (70kPa O_2, 10kPa CO_2) and the lowest amount was observed in arils stored in the normal atmosphere at the end of storage (Belay *et al*., 2017 a). Increasing synthesis of VOCs in response to wound (Amaro *et al*., 2012), or high CO_2 concentration leads to disruption of enzymatic systems, such as the lipoxygenase pathway (Giuggioli *et al*., 2015) which catalyzes the oxidation of unsaturated fatty acids.

Microbial load

Fungi (yeasts and molds) are important pathogenic microorganisms that are resistant to acid conditions (Jacxsens *et al*., 2001; Firdous *et al*., 2023). Yeasts are facultative anaerobes, and in contrast, molds are aerobes, which has been observed high CO_2 (>10%) inhibits mold growth (Molin, 2000). The reduction count of mesophilic bacteria has been reported in minimal processing pomegranate cv. Hicaznar under a high O_2 atmosphere (70 kPa) compared to low O_2 and normal atmosphere at 5°C (Ayhan and Estürk, 2009). A high O_2 atmosphere is used in fresh-cut due to its ability to prevent anaerobic fermentation, enzymatic discoloration and micro-

bial growth (Jacxsens *et al*., 2001) and it is effective by increasing the lag phase of growth and reducing the growth of bacteria and yeast in arils pomegranate (Belay *et al*., 2017 a; Moradinezhad *et al*., 2020). The inhibitory effect of the high O_2 is due to the toxicity of oxygen, which causes damage to the antioxidant system, DNA and nucleoproteins of microorganisms by ROS (O^{2-}, H_2O_2 and OH^-) produced at a partial pressure of O_2 (Tomas-Callejas *et al*., 2011). Pre-storage short-term high CO_2treatment significantly reduced the decay of pomegranate fruits during cold storage (Moradinezhad *et al*., 2018). High CO_2 reduces the microbial load of fruit by penetrating the microbial membrane, changing intracellular pH or forming carbonic acid, which has bacteriostatic effects (Zhang *et al*., 2013 b; Banda *et al*., 2015; Belay *et al*., 2017 b; Ranjbari *et al*., 2018; Van de Velde *et al*., 2019, 2020; Moradinezhad and Dorostkar, 2020). High CO_2 pretreatment has significant potential to prevent water loss, oxidative damage, and control decay. It seems to be related to the induction of specific defense proteins, including dehydrins and pathogenesis-related proteins, as well as endogenous protective osmolytes (Vazquez-Hernandez *et al*., 2018). At ambient air temperature, the active modified atmosphere affected on the chemical and qualitative characteristics of pomegranate arils, which were related to the reduction of microbial load, safety and high organoleptic properties (Rokalla *et al*., 2022).

3. Conclusions and future prospects

Post-harvest loss is one of the main problems in the pomegranate industry worldwide. Since the quality of the fruit is determined by internal and external characteristics, it is necessary to maintain the overall quality of the product for supply to consumers. Considering that pomegranate fruit is non-climacteric, the use of MAP polymer films may have a good potential for the maintenance of its quality.In this study, the mechanism of the effects of MAP on the physicochemical and qualitative characteristics of whole and minimally processed arils of pomegranate werereviewed, and indicated that MAP had a significant effect to prevent chilling injury and maintain fruit quality. Several studies have reported the advantages of the modified atmosphere in extending the shelf life based on low O_2 concentration and enriched CO_2. MAP and vacuum packaging comprising optimal concentrations O_2 and CO_2depends on physiology cultivar is a valuable technique to maintain nutritional quality, and antioxidant activity, reduce weight loss, and control the storage diseases and disorders of whole pomegranate fruit and arils.However, there is a need for extensive studies to develop the MAP system for pomegranate arils and fruit in different commercial cultivars.

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Review paper

AHS
Advances in Horticultural Science

A review: Molecular identification of orchid mycorrhiza

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Key words: Identification, mycorrhizal, orchid.

Abstract: Orchids are a diverse and widespread family of flowering plants, with over 25,000 known species and more than 100,000 hybrids and cultivars. Orchids are characterised by their often showy and highly specialised flowers and have unique and intricate floral. Orchids are known to be highly dependent on their mycorrhizal fungi for nutrient uptake, especially during the early stages of their development. Orchid seeds lack the endosperm present in most other seeds, which means they cannot germinate without a source of nutrition. The relationship between orchids and mycorrhiza is known as orchid mycorrhizae or orchid mycorrhiza. In orchid mycorrhiza, the orchid plant forms a mutualistic relationship with certain species of fungi that are able to penetrate the orchid's roots and colonise its tissues to provides the orchid with essential nutrients. Orchid mycorrhizal fungi are often highly specific, meaning that they can only form partnerships with certain orchid species, and vice versa. The importance of mycorrhizal fungi in the orchid life cycle is crucial from both evolutionary and ecological standpoints. Therefore, it is essential to acquire a thorough comprehension of this relationship and develop methodologies for isolating, identifying, and preserving significant fungal strains that are associated with different orchid species. In recent years, there has been a considerable increase in research concentration on mycorrhizal interactions in orchids. However, certain inquiries remain unresolved pertaining to the fungal communities associated with orchids as well as the divergences notices across different species and geographical locales. The present paper provides a through, and extensive analysis of the fungal life associated with orchids. This article presents a succinct overview of the molecular techniques utilised by researchers globally to isolate and identify peloton-forming fungi in both temperateterrestrial and tropical orchids. The review begins by proving a concise introduction to the background material regarding the wide range of fungal species that are linked with orchids. It then proceeds to explores the topic of orchid mycorrhizal fungi (OMF) and orchid non-mycorrhizal fungi (ONF). The subsequent analysis explores the crucial function that orchid mycorrhizal fungi play in the processes of seed germination and development. Moreover, the study elaborates on the methodologies utilised for isolating fungi, extracting fungal DNA, selecting primers, amplifying DNA and subsequent analysis sequence data. This article considers several molecular identification approaches that are used in studying orchid endophytic mycorrhizal. Using molecular approaches, orchid mycorrhizal can be further explored and identified.

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

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1. Introduction

The Orchidaceae family is considered the second largest among flowering plants, with its size exceeded only by the Asteraceae family (Givnish *et al.,* 2015). According to Govaerts *et al*. (2017), the number of recognised orchid species is estimated to be 29,199. The decision to classify all orchids under Appendices I and II by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2017 effectively prohibited the illicit trade of these plants (Hinsley *et al*., 2018). Based on the evaluations made on a total of 1770 species of orchids, it has been determined that approximately 46.5% of these species are classified under the categories of vulnerability, endangered, or critical endangered as reported by the International Union for Conservation of Nature (IUCN, 2021). This precarious state remains due to a variety of variables, including their difficult germination process and human intervention, such as overcollection caused by economic and horticultural needs (Pujasatria *et al*., 2020; Suresh *et al.,* 2023).

Orchids are extraordinarily important for biodiversity, conservation, and producing a vast array of therapeutic substances, nutritious foods, and ornamental plants (Hinsley *et al.,* 2018). Orchid conservationists strive to manage market needs and biodiversity on a global scale, which would need large-scale production (Pujasatria *et al*., 2020). Numerous species encounter the peril of extinction; however, orchids adopt two distinct evolutionary strategies, namely sympodial growth and monopodial development, which are regulated by a diverse array of endophytic fungus species. These techniques serve to extend the longevity of orchids as herbaceous plants. (Srivastava, 2018). Orchid endophyte has a different way of penetrating and colonising their host, which makes them different from another fungal pathogen. For example, orchid fungi endophytes enter through stomata laterally in the anticlinal epidermal cell. They remain intracellularly in the shoot without colonising the cell. In contrast, pathogen fungi enter directly from the cell wall and typically grow extracellularly, potentially causing harm to the host (Sarsaiya *et al*., 2019).

Diverse fungal taxa include mutualistic mycorhiza, endophytic fungi and considerably diverse as well as non-mycorrhizal fungal associates. The role of the root-allied fungi is not well understood. According to Lee and Yeung (2018), some of these fungi may supply organic carbon, nutrients, and water to the orchid, but the degree of this transfer is typically unknown. Numerous report on specific mycorrhizal fungi also shows the ability to stimulate the embryo's development and supply it with necessary nutrients, allowing the orchid seeds to germinate (Liu *et al*., 2010; Zhang *et al*., 2016; Shao *et al*., 2017; Herrera *et al*., 2019; Shah *et al*., 2019; Suresh *et al.,* 2023).

In recent years, the has been a significant transformation in the application of molecular techniques. The identification of fungi within roots has been accomplished through the application of polymerase chain reaction (PCR) techniques, employing fungal-specific primers (Gardes and Bruns, 1993). Such methods have been used to characterize mycobionts of Orchidaceae, Taylor and Bruns (1999) employed these techniques to characterise mycobionts of Orchidaceae, thereby removing the laboratories process of culturing. The region that is most frequently studied is the nuclear ribosomal internal transcriber spaces (ITS). Therefore, there is a want for molecular techniques capable of discerning distinct fungal species in cases where numerous fungal species are present in a single plant.

The present review has provided an overview of the principal discoveries and methodologies utilised in the discipline, underscoring the significance of molecular techniques such as fungal DNA extraction, primer selection, polymerase chain reaction (PCR), and high throughput sequencing (HTS) in discerning the taxonomy of mycorrhizal fungi and elucidating the underlying molecular mechanisms that regulate these symbiotic relationships. Furthermore, the utilisation of molecular method has provided researchers with enhanced capabilities to explore the extensive range of orchid mycorrhizal variety. The investigation has not only shown evolutionary relationship but has also yielded significant insights into ecological and conservation concerns. A thorough comprehension of mycorrhizal connections is essential for the efficient preservation of orchid species. In addition, the exploration of orchid mycorrhizal fungus in the fields of biotechnology and agriculture has resulted in the identification of new and important mycorrhizal fungi. In the context of identifying orchid mycorrhizal fungi, many methodologies are routinely applied, encompassing the isolation and cultivation of fungi, microscopic analysis and molecular studies. The ongoing refining

and improvement of these techniques play a crucial role in further our understanding and fascinating associations between orchids and their mycorrhizal fungus.

2. Orchid and its fungi diversity

Orchids form a unique symbiotic relationship with the plant and animal species present in forest habitats in order to acquires nutrients, facilitate their own development, and facilitate the process of pollination. The mycorrhizal fungi, which exhibit symbiotic germination, are if significant importance in facilitating embryo development and supply vital nutrients within the natural environment. This symbiotic relationship is crucial in the effective germination of orchid seeds (Liu *et al*., 2010; Herrera *et al*., 2019). Fungi have a crucial role as the principal provider of essential nutrients for developing Orchidaceae plants, especially in setting characterised by low nutrients availability (Long *et al*., 2022).

Orchids interact with a smaller number of mycorrhizal fungi than other mycorrhizal plants, with greater specificity for orchid mycorrhizal fungi than ectomycorrhizae, arbuscular mycorrhizae, and even ericoid mycorrhizal fungi. In symbiotic connection, fungi provide plants with water and mineral nutrients (especially phosphorus) while protecting them from biotic and abiotic stresses. In exchange, plant hosts provide carbon from photosynthesis to the fungi (Rasmussen, 1995; Tedersoo *et al*., 2017).

Many orchid species cannot commence germination or grow without their compatible symbiotic fungus (Rasmussen, 1995; Davis *et al*., 2015; Fay, 2018; Attri, 2022), as their specificity of mycorrhizal connections that permit *in situ* symbiotic seed germination in orchids is frequently so rigorous. The aforementioned circumstance has stimulated inquiries into the importance of fungus in symbiotic relationships that are equally crucial and beneficial for the *ex-situ* preservation of orchids, specifically in the context of reintroduction endeavours. The first recorded evidence of a mycorrhizal fungus in an orchid may be traced back to the year 1824, as documented by the renowned German naturalist Heinrich Link. Nevertheless, the specific function of the fungus remained ambiguous until the early 1900s when Nöel Bernard established a scientific correlation between filamentous fungi and the process of seed germination (Arditti and Pridgeon, 1997). Following this, in the early 1900s, the study of orchid endophytes emerged as a significant area of interest within the field of orchid biology research. Chand *et al*. (2020) conducted a comprehensive investigation wherein they isolated and identified many orchid endophytes, and thoroughly evaluated their probable role in orchid symbiosis.

Orchids frequently establish symbiotic relationships with fungus that display substantial evolutionary and ecological variability. Epiphytic orchids exhibit a prevalence of both Basidiomycota and Ascomycota in their aerial roots as well as subterranean roots or rhizomes, while Chytridiomycota, Glomeromycota, Zygomycota, or Mucoromycota are present in comparatively smaller quantities (Waud *et al*., 2014; Cevallos *et al*., 2017; Egidi *et al*., 2018; Novotná *et al*., 2018). The classification of orchid fungus is determined by the existence or absence of functional pelotons within cortical cells, leading to the categorization of orchid mycorrhizal fungi (OMF) or orchid non-mycorrhizal fungi (ONF) (Li *et al*., 2021).

3. Orchid mycorrhizal fungi (OMF) and Orchid non-mycorrhizal fungi (ONF)

The phenomenon referred to as "orchid mycorrhiza" pertains to the symbiotic relationship established between the orchid plant and many fungal species that are capable of cohabiting within its root system. The germination of an orchid mycorrhizal fungus (OMF), and these seeds rely on one or more OMF's for sustenance during their whole life (Bidartondo and Read, 2008). Within the realm of fungi, a subset of these organisms can be classified as transient, denoting their inability to maintain a sustained presence within the developing and maturing tissues of orchids. Conversely, there exist other fungi that establish more long-lasting associations with these plants. According to Lee and Yeung (2018), during the maturation process of orchids, specific fungi that play a role in facilitating germination persist as "permanent residents" whereas other fungi initiate germination and are subsequently replaced by different fungal partners.

The identification of coiled pelotons within cortical root cells is recognised as a characteristic feature of orchid mycorrhizal fungus (OMF), as examiner in research undertaken by Dearnaley *et al.* (2016) as well as Rasmussen (1995). In contrast,

orchid mycorrhizal fungi (ONF) pertain to a distinct classification of endophytic fungi that reside within the roots or other tissues or orchids at specified phases of their life cycle. Nevertheless, it is imperative to elucidate that oligotrophic nitrogen-fixation bacteria (ONFs) are devoid of peloton-like structures and do not elicit any noticeable pathogenic consequences in the host plants. The aforementioned phenomenon has been emphasised in scientific inquiries conducted by Sisti *et al*. (2019) and Selosse *et al*. (2018).

Several investigations, like those conducted by Herrera *et al*. (2019) and Waterman *et al*. (2011), have shown empirical evidence indicating the involvement of certain orchid mycorrhizal fungi (OMFs) in the process of decomposition. The observed mycorrhizal fungi (OMFs) have been documented to facilitate the decomposition of nearby substrates and provide essential nutrients to orchids. It is important to acknowledge that specific obligatory mycoheterotrophic fungi (OMFs) may have experienced evolutionary shifts from ancestral obligate non-photosynthetic fungus (ONFs), gradually developing mycorrhizal capacities. The aforementioned phenomenon has been thoroughly investigated in scholarly studies conducted by Selosse *et al*. (2018) and Wang *et al*. (2021).

The classification of orchid mycorrhizal fungus (OMF) has a wide range of fungal species, consisting of at least 17 families from the basidiomycetes group and five families or genera from the ascomycetes group, as documented by Dearnaley (2007) and Dearnaley *et al*. (2012). Within this set, there are several noteworthy groups, namely Ceratobasidiaceae (Cantharellales), Tulasnellaceae, and Serendipitaceae, which were previously referred to as the Sebacinales clade B. The classification of these groupings as Rhizoctonia-type Basidiomycetes is largely acknowledged in the scientific community, as evidenced by multiple research (Rasmussen, 1995; Bayman and Otero, 2006; Sisti *et al*., 2019; Selosse *et al*., 2018; Jędryczka *et al*., 2023). Basidiomycetes and Ascomycetes, which are widely distributed in terrestrial ecosystems and cultivated plants globally, have notable associations with orchids (Trivedi *et al*., 2020; Wang *et al*., 2019).

The significance of orchid mycorrhizal fungi (OMF) in specific microenvironments cannot be understated, as they play a crucial role in promoting the germination of orchid seeds and the subsequent growth of orchid seedlings. As a result, geographical areas that display a significant occurrence of orchid mycorrhizal fungi (OMF) tend to showcase a higher range of orchid species, as documented by Li *et al*. (2021). In their study, Hemrová *et al*. (2019) conducted germination tests and developed species distribution models that integrated multiple habitat parameters. The results of their study emphasized the crucial significance of fungal symbionts in influencing the spatial distribution of orchids on a large geographical scale. Furthermore, McCormick *et al.* (2019) and other scientific inquiries have provided substantial data supporting a strong and positive association between the prevalence of mycoheterotrophic orchids, which depend on fungi for nourishment, and the existence of OMF. The cumulative evidence suggests that OMF has a significant role in shaping the population dynamics of orchids.

4. Orchid mycorrhizal and its roles in seed germination and development

In general, asymbiotic or symbiotic procedures can be used to germinate orchid seeds (Yam and Arditti, 2009). It has been demonstrated that asymbiotic seed germination is an effective method for producing plantlets of numerous orchid species for both commercial and conservation.

It was believed that root orchid mycorrhizal fungi are the actual source of seed-germinating orchid mycorrhizal fungi (Rasmussen, 1995). Root fungal endophytes are seen as advantageous plant residents that may increase their productivity and eventually support ecological functions. Roots of mature plants have provided fungi that have been isolated and tested, with several successes have been attained employing these fungi (Nontachaiyapoom *et al*., 2011; Sebastián *et al*., 2014). The *in situ*/*ex-situ* seed baiting technique has been increasingly popular in recent years as a means of obtaining efficacious fungi that facilitate seed germination. According to previous studies conducted by Zhou and Gao (2016) and Rasmussen and Whigham (1993), it has been observed that fungus obtained from naturally occurring protocorms or seedlings possess the capacity to induce seed germination and facilitate the subsequent development of seedlings (Table 1). Shao *et al*. (2020) conducted a conservation project with the objective of protecting *Dendrobium* species

Table 1 a - List of orchid mycorrhizal fungi that had been identified and their roles in orchid micropropagation

Orchid	Fungi *sp.*	Roles	References
Vanda wightii (E)	*Ceratobasidium sp.*	Seed germination	Suresh *et al.* (2023)
Paphiopedilum barbigerum (T)	*Epulorhiza sp.*	Seed germination and seedling development	Tian *et al.* (2022)
Serapias vomeracea (T)	*Tulasnella calospora*	Seed germination and seedling development	Ghirardo *et al*. (2020)
Epidendrum secundum (T)	*Ceratobasidium* sp., *Sebacina vermifera*	Seed germination and seedling development	Durán-López *et al.* (2019)
Dactylorhiza majalis (T)	*Piriformospora indica*	Seed germination	Shah *et al*. (2019)
Chloraea gavilu (T)	*Tulasnella* sp.	Seed germination	Herrera *et al.* (2017)
Aerides multiflora (E)	*Ceratobasidium* sp.	Seed germination	Bhatti *et al*. (2017)
Dendrobium friedericksianum (E)	*Tulasnella* sp., *Tulasnellaceae Rigidoporus vinctus, Polyporales Ceratobasidium* sp., *Tulasnellaceae Flavodon flavus, Polyporales Nigroporus vinosus, Polyporales Coriolopsis retropicta, Polyporales Valsa eugeniae, Diaporthales.*	Seed germination and seedling development	Agustini *et al.* (2016)
Paphiopedilum villosum (E)	*Tulasnella* sp., *Tulasnellaceae*	Seed germination	Khamchatra *et al*. (2016)
Dendrobium lancifolium (E)	*Rhizoctonia* sp.	Seed germination	Agustini *et al.* (2016)
Liparis japonica (T)	*Rhizoctonia* sp.	Seed germination	Ding *et al*. (2014)
Dendrobium aphyllum (E)	*Tulasnella* sp., *Trichoderma* sp.	Seed germination	Zi *et al.* (2014)
Dendrobium aphyllum (E), *Dendrobium devianum* (E), *and Cymbidium manni* (E)	*Tulasnella* sp., *Epulorhiza sp.*	Seedling growth	Zi *et al.* (2014)
Dendrobium officinal (E)	*Tulasnella* sp.	Seed germination and seedling growth	Ming *et al.* (2014)
Dendrobium nobile (E), *Dendrobium Chrysotoxum* (E), *Dendrobium falconer* (E), *Dendrobium aphyllum* (E)	*Xyalariaceae* sp.	Seed germination	Chen *et al*. (2013)
Dendrobium crumenatum (E)	*Guignardia endophyllicola*	Seed germination	Mangunwardoyo *et al*. (2011)
Pecteilis susannae (L.)	*Epulorhiza* sp.	Seed germination and development	Chutima *et al*. (2011)

E= epiphytes; T= Terrestrial.

Table 1 b - List of orchid mycorrhizal fungi that had been identified and their roles in orchid micropropagation

Orchid	Fungi *sp.*	Roles	References
Dendrobium nobile (E)	*Leptodontidium*	Seedling development	Hou and Guo (2009)
Cymbidium eburneum (E)	*Alternaria sp., Chaetomium sp, Fusarium sp.*	Vegetative growth	Zhao and Liu (2008)
Gastrodia elata (T)	*Mycena osmundicola*	Seed germination	Kim *et al*. (2006)
Cymbidium goeringii (T)	*Rhizoctonia* sp	Seedling development	Jianrong *et al*. (2005)
Gastrodia elata (T)	*Mycena osmundicolor*	Seed germination	Hong *et al.* (2002)
Paphiopedilum armeniacum (T)	*Phacodium* sp.	Seedling development	Ming and Zhou (2001)
Cypripedium reginae (T)	*Fusarium* sp.	Seed germination	Warcup (1981)
Dendrobium discolor (E),	*Tulasnella cruciate, Tulasnella irregularis, Tulasnella allantospora*	Seed germination	Warcup (1981)
Calochilus sp. (T), *Diuris maculata Sm.* (T), *Spiranthes sinensis* (T)	*Tulasnella asymmetrica, Tulasnella cruciate, Tulasnella irregularis, Tulasnella violea, Tulasnella allantospora*	Seed germination	Warcup (1981)
Diuris sulphurea. R.Br. (T)	*Tulasnella asymmetrica*	Seed germination	Warcup (1981)
Orthocersa strictum (T)	*Tulasnella asymmetrica, Tulasnella cruciate, Tulasnella irregularis, Tulasnella violea*	Seed germination	Warcup (1981)
Thelymitra ixoides (T)	*Tulasnella asymmetrica, Tulasnella cruciata*	Seed germination	Warcup (1981)
Thelymitra flexuosa (T)	*Tulasnella irregularis, Tulasnella cruciata*	Seed germination	Warcup (1981)
Thelymitra media (T)	*Tulasnella violea, Tulasnella asymmetrica*	Seed germination	Warcup (1981)
Thelymitra carnea (T)	*Tulasnella allantospora, Tulasnella violea*	Seed germination	Warcup (1981)

E= epiphytes; T= Terrestrial.

that have been excessively harvested. They effectively isolated and obtained fungi that enhance germination for several *Dendrobium* species using the seed baiting approach, as described by Huang *et al*. (2018).

5. Fungal DNA extraction

There are various methodologies commonly employed for the isolation of orchid mycorrhizal fungus from orchid plants. These methodologies encompass the isolation of complete tissue or tissue segments, *in situ* seedings, trapping isolation, and isolation from a solitary peloton. Among these methods, the technique of isolating a single peloton, which involves micromanipulation-based isolation from host cells, is widely regarded as the most reliable and precise approach for extracting endophytic mycorrhizal fungi (Zettler *et al*., 2003; Batty *et al*., 2006; Zi *et al*., 2014; Zettler and Corey, 2018). The prevailing conventional method for molecular identification of orchid mycorrhizal fungus generally entails the extraction of DNA from agar plates or liquid cultures, as opposed to direct extraction from orchid roots (Zettler and Corey, 2018).

The fungal cell wall primarily consists of around 80-90% polysaccharides, inorganic ions, lipids, polyphosphates, and proteins, which together form the matrix that binds the wall. This type of cell wall also can be characterized by microfibrillar components like chitin, β-glucan, and/or cellulose, which pose challenges in DNA extraction (Turzhanova *et al*., 2018). Moreover, the presence of a substantial quantity of secondary metabolites, such as melanin, can impede subsequent reactions (Fernandez *et al*., 2016; Janowski *et al*., 2019) This become a major challenge in DNA extraction of fungi as it has a robust cell walls that are resist to lysis method (Jiang *et al*., 2011). The isolating nucleic acids from fungi, often necessitates the incorporation of additional lysis steps, which can include enzymatic lysis, mechanical homogenization, sonication, or the use of potentially harmful chemicals (Turzhanova *et al*., 2018).

DNA samples were gathered over a period of 15 years, during which a diverse range of extraction procedures were utilised to extract fungal DNA. Nevertheless, the extraction of DNA from the various types of fungi encountered does not have a universally optimised approach. The standard procedure for the extraction of fungal DNA typically encompasses several sequential stages. These stages involve the cultivation of fungi in either liquid or solid growth media, disruption of the fungal cell wall, elimination of proteins using phenol and chloroform, and subsequent isolation of DNA through precipitation with ethanol or isopropanol (Faggi *et al.,* 2005). Even though the presence of polysaccharide and polyphenolic compound in the fungi may inhibit the activity and effect of DNA polymerase, but they can be easily removed by either using a vacuum or spin column and by mixing the sample with bovine serum albumin (BSA), β-mercaptoethanol (βME), N-trimethyl ammonium bromide (CTAB) and Polyvinylpyrrolidone (PVP) (Tripathy *et al*., 2017).

A variety of methodologies have been devised to isolate DNA from fungal tissues, and the most efficacious DNA extraction procedures frequently integrate physical methodologies (such as microwave treatment, freeze/thaw cycles, homogenization using glass beads, and grinding in liquid nitrogen) with enzymatic approaches (including gluconases, chitinases, and proteases) (Zhang *et al*., 2010). The exists variety of ways for extracting DNA and among them, the CTAB approach (Gardes and Bruns, 1993) is frequently utilised.

Additional alternatives for fungal genomic DNA isolation kits are the Omega Fungal E.Z.N.A kit (manufactured by Omega Biotech, Doraville, GA, USA), the Qiagen Plant DNeasy kit, Genomic Tip kits (Qiagen, Valencia, Cam USA), or Sangin Biotech Rapid Fungi Genomic DNA isolation kits (Long *et al*., 2022). In order to ascertain the effectiveness of a DNA extraction technique, it is important to evaluate both the quality and quantity of the DNA obtained. The concentration of DNA in the samples was assessed by employing spectrophotometry at wavelength of 260 nm, with measurement expressed in units of nanograms per microliter (ng/µL). In addition, the assessment of DNA purity was conducted by determining the A260/A280 ratio and A260/280 ratio utilising either a UV-VIS spectrophotometer or Nanodrop devise (Thermo Electron Scientific Instruments LLC, USA). Generally, the A260/A280 ratio exceeded 1.8 suggesting that the DNA was largely devoid of proteins. In terms of the A260/A230 ratio, if it was approximately 2, that indicate the samples did not contain significant impurities such as carbohydrates, peptides, phenols, salts, or aromatic compounds (Turzhanova *et al*., 2018). Furthermore, the quality of the DNA also can be evaluated through

electrophoresis after PCR amplification of the genomic DNA, using gene-specific primers (Tripathy *et al*., 2017).

The standard CTAB phenol-chloroform extraction procedure has proven effective across a wide range of species (Strugnell *et al*., 2006; Reineke *et al*., 1998) and produce a high purity of DNA (Zettler and Corey 2018). Study by Turzhanova *et al*. (2018) on optimization of DNA extraction methods of fungi has shown that CTAB-method and DNeasy Plant mini Kit (Qiagen) resulted a highest DNA quality, while SDS method resulted in the lowest sample yields and quality. However, CTAB-method uses toxic chemicals and requires a significant amount of bench time, both limiting its applicability when scaling up for big comparative research (Schiebelhut *et al*., 2017). Nowadays, commercial DNA extraction kits are more desirable since they reduce exposure to toxic chemicals and allow for faster extraction periods. These kits could offer a range of low- to high-throughput processing, vary in price from quite inexpensive to highly costly, and may require some specialist gear. Table 2 below shows a list of extraction methods and kits used in the extraction method of DNA orchid fungi.

A large percentage of orchid mycorrhizal fungi are mycelia sterilia. Conventional techniques have led to a paraphyletic taxonomy in which unrelated fungi are grouped together, requiring molecular techniques for accurate identification, phylogenetic inference, and genetic relatedness (Sen *et al*., 1999; Otero *et al*., 2002; Shan *et al*., 2002; Yagame *et al*., 2008). Molecular sequencing, microscopic examination, and biochemical analysis were among the most used methods to identify mycorrhizal fungi. For fungi identification by morphological characterisation, it

Table 2 - Types of manual DNA extraction protocol used to extract mycorrhizal fungi DNA

Protocol name	Abbreviation	Chemistry/mechanism	Kits/supplies required	DNA extraction time	References
Cetytrimethyl ammonium bromide (CTAB)-Phenol-chloroform	CTAB	CTAB lysis, followed by phenol chloroform purification step	All reagents are made in-house	1 hour 30 min	Sambrook *et al.* (2001); Dawson *et al*. (1998)
Sodium Dodecyl Sulfate	SDS lysis, followed by phenol chloroform purification step	SDS and mercaptoethanol lysis, followed by chloroform purification step	All reagents are made in-house	1 hour 5 min	Turzhanova *et al*. (2018)
Phenol Chloroform Isoamyl alcohol extraction method	PCI	Buffer lysis. Followed by Phenol/chloroform /isoamyl alcohol purification step	All reagents are made in-house	2 hour 10 min	Varma and Kwon Chung (1991)
EZNA SP Fungal DNA	Omega Fungal EZNA	Silica based purification system	Omega Fungal EZNA kit (Omega Biotek, Doraville, GA, USA)	45 min	Omega (2019)
Qiamp Mini Kit (Qiagen)	QIAamp Mini kit	Silica based purification system	Qiamp Mini Kit (Qiagen)	35 min	Turzhanova *et al*. (2018)
Fungi/Yeast Genomic DNA Isolation (Norgen)*	Fungi/Yeast Genomic DNA Isolation	Silica based purification system	Fungi/Yeast Genomic DNA Isolation Kit (Norgen)	More than 2 hours	Kumar and Mugunthan (2018)

* Modified method.

can be conducted alone or in combination with molecular analysis, and usually, most research will use both combination methods in identifying mycorrhizal fungi. However, the orchid fungus is notoriously difficult to be determined at the species level because they do not sporulate readily on cultures (Boddington and Dearnaley, 2008; Ko *et al.*, 2011; Ma *et al*., 2015).

6. Primer selection for fungal amplification

After the completion of DNA extraction from orchid mycorrhizal fungi, the subsequent step involves the amplification of fungal DNA through the utilisation of a polymerase chain reaction (PCR) technique. This amplification process necessitates the use of primers that are specifically designed to target the ribosomal DNA (rDNA) region. The rDNA cluster consists of several components, including 18S rDNA, 5.8S rDNA, 28S rDNA, the External Transcribed Spacer (ETS), and Internal Transcribed Spacer 1 and 2, which are generally referred to as ITS1 and ITS2. The utilisation of the ITS region for molecular identification of fungi can be traced back to the early 1990s, as shown by Horton and Bruns (2001) and Seifert (2009).

The utilisation of the ITS region for molecular identification is of great significance in fungal identification, principally owing to the inclusion of two remarkably variable spacers, namely ITS1 and ITS2, which frequently exhibit species-specific characteristics either independently or in conjunction. Moreover, it includes the 5.8S gene, which is renowned for its exceptional level of conservation. The high degree of sequence conservation observed in the adjacent genes, along with their designation as the region undergoing the most rapid evolution and the existence of multiple copies of the ribosomal operon, facilitates the efficiency of primer design and PCR amplification for the ITS region (Bengtsson Palme *et al*., 2013; Fajarningsih, 2016; Raja *et al*., 2017). These two spacers are copied from the ribosomal DNA, and when the ribosomal RNAs complete, they are removed from the rRNAs. Since the spacers are not used in the final structure of the ribosome, they are not strongly selected against mutations. Therefore, the identification of mycorrhizal fungus is considered efficient by using a region-specific to eukaryotes (Tedersoo and Nilsson, 2016).

The nuclear ribosomal RNA genes, including the small subunit (SSU) (18S) and large subunit (LSU) (28S) are commonly utilised in scientific investigations pertaining to aquatic fungus and arbuscular mycorrhizal fungi. Nevertheless, in the case of ascomycetes and basidiomycetes, these markers generally offer taxonomic insights primarily at level beyond the species, and occasionally at the genus level. This problem is caused by the fact that the SSU and LSU sequences of the many species that belong to these fungal groupings have only minute to nonexistent differences between them. Because of this, precise distinction becomes a challenging obstacle. According to the findings of the research carried out by Nilsson *et al*. (2019), the ability of SSU, LSU, and protein-coding genes like the RNA polymerase gene RPB2 to be aligned across different fungal phyla is a significant benefit offered by these types of genes. This makes it possible to analyse large-scale phylogenetic relationships at the phylum and order levels, which is something that the ITS region normally has difficulty accomplishing without very identical reference sequences (Větrovský *et al*., 2016). Because the ITS region often ranges in length from 500-700 bases, the majority of high-throughput sequencing (HTS) studies concentrate on the shorter ITS1 or ITS2 subregions, which typically range in length from 250-400 bases. This constraint is the result of the fact that the ITS region is normally between 500-700 bases in length. According to Tedersoo *et al*. (2015), the ITS2 subregion in particular stands out due to the fact that it exhibits lesser length fluctuations and more universal primer sites. This, in turn, results in reduced taxonomic bias.

The ITS1 and ITS2 subregions have demonstrated their suitability for second generation High-Throughput Sequencing (HTS) techniques. However, third generation methodologies, such as those utilising PacBiosciences (PacBio) and Oxford Nanopore platforms, provide the ability to target the complete ITS region, as well as segments or even the entire adjacent rRNA genes (Nilsson *et al*., 2019). Targeting the entire Internal Transcribed Spacer (ITS) area, rather than its subregions has several advantages, including improved taxonomic accuracy and less amplification of non-viable organism. Nevertheless, one limitation of this methodology is its reduced efficacy when utilised on materials of subpar quality, such as ancient herbarium specimens, which degrade to a degree where doing ITS DNA sequencing becomes impractical (Tedersoo *et al*.,

2017). According to study conducted by Nilsson *et al*. (2019), it is recommended to allocate a significant amount of effort to the analysis and selection of primers, this is due to the fact that only a limited number of primers have the capability to amplify over 90% of fungal groups. Additionally, the process of primer selection necessitates meticulous examination of the target taxa, as highlighted by Tedersoo *et al*. (2015). The following table 3 and 4 show an illustrative depiction of ITS primers together with their corresponding sequences.

7. Identified fungal from orchid root by using internal transcribe region

Gardes and Bruns (1993) and White *et al*. (1990) have produced well recognised primers in the field of fungal ecology for species-level identification based on sequencing. These primers, namely ITS1, ITS2, ITS3, ITS4, ITS1F, ITS86F, and cNL2f, are considered to be broad-spectrum primers. The ITS1 and ITS4 primers are commonly employed as standard primers in numerous laboratories (Fajarningsih, 2016). The list of endophytic fungi that have been isolated and identified from orchid roots is presented in table 5. This was accomplished by employing a broad-spectrum primer (ITS1 and ITS4).

However, some primers are designed to be specific. For example, the ITS86F primers are used primarily for medically important fungal pathogen, but they are rarely used in mycorrhizal identification, especially fungi communities from environmental samples. In orchid mycorrhizal fungi identification, the ITS1-F is one of the most effective primers for the ITS region amplification, especially for Eumycota. For example, the primer ITS1-F and ITS4 always used in pair to identified an *Fusarium* sp. as in the study by Sukarno *et al.* (2023), where they manage to identified several species of *Fusarium* using this primer combination. The ITS1-F and ITS4-B primer were designed to be specific basidiomycetes (Gardes and Bruns, 1993). Besides that, both primers can minimise plant sequence amplification (Taylor and McCormick, 2008). However, this primer is ineffective in amplifying some species of Tulasnellaceae that belong to Basidiomycota phylum, as their nuclear ribosomal is evolving rapidly and some primers are typically conserved along the Eumycota are not maintained in Tulasnellaceae

Table 3 - List of recommended primer for identification of orchid mycorrhizal

Primer	Sequence (5'-3')	References
Modified ITS1ngs	TCCGTAGGTGAACCTGC	Oja *et al*. (2014)
Modified ITS1Fngs	GGTCATTTAGAGGAAGTAA	Oja *et al*. (2014)
Modified ITS4ngs	TCCTSCGCTTATTGATATGC	Oja *et al*. (2014)
ITS4Tul2	TTCTTTTCCTCCGCTGAWTA	Oja *et al*. (2014)
TW14ngs	CTATCCTGRGRGAAAYTTC	Tedersoo *et al*. (2014)
fITS9	GAACGCAGCRAAIIGYGA	Ihrmark *et al*. (2012)
gITS7	GTGARTCATCGARTCTTTG	Ihrmark *et al*. (2012)
fITS7	GTGARTCATCGAATCTTC	Ihrmark *et al*. (2012)
ITS4Tul	CCGCCAGATTCACACATTGA	Taylors and McCormick (2008)
ITS1-OF	AACTCGGCCATTTAGAGGAAGT/AACTTGGTCATTTAGAGGAAGT	Taylors and McCormick (2008)
ITS4-OF	GTTACTAGGGGAATCCTTGTT	Taylors and McCormick (2008)
ITS86F	GTGAATCATCGAATCTTTGAA	Turenne *et al*. (1999)
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
ITS4B	CAGGAGACTTGTACACGGTCCAG	Gardes and Bruns (1993)
ITS1	TCCGTAGGTGAACCTGCGG	White *et al*. (1990)
ITS2	GCTGCGTTCTTCATCGATGC	White *et al*. (1990)
ITS3	GCATCGATGAAGAACGCAGC	White *et al*. (1990)
ITS4	TCCTCCGCTTATTGATATGC	White *et al*. (1990)
NS7	GAGGCAATAACAGGTCTGTGATGC	White *et al*. (1990)
cNL2f	GTTTCCCTTTTAACAATTTCAC	White *et al*. (1990)

Table 4 - List of recommended primer pair for sequencing of orchid mycorrhizal partners

Primer pair	Primer name (forward/ reverse)	Sequence (5'-3')	Target region	Annealing temp (°C)	Target clade (orchid specific group fungi)	References
ITS1/ITS4	ITS1 (F) ITS4 (R)	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	ITS1/ITS2	53	All Basidiomycota	White *et al*. (1990)
ITS1/ ITS4- Tul	ITS1 (F) ITS4-Tul	TCCGTAGGTGAACCTGCGG CCGCCAGATTCACACATTGA	ITS1/ITS2	54	*Tulasnella*	Taylor and McCormick (2008)
ITS1-OF/ITS4-OF	ITS1-OF (F) ITS1-OF (F) ITS4-OF (R)	AACTCGGCCATTTAGAGGAAGT AACTTGGTCATTTAGAGGAAGT GTTACTAGGGGAATCCTTGTT	ITS1/ITS2	60	All Basidiomycota	Taylor and McCormick (2008)
SSU1318-Tom/LSU-Tom4	SSU1318-Tom (F) LSU-Tom4	CGATAACGAACGAGACCTTAT GCCCTGTTCCAAGAGACTTA	SSU/LSU	62	Thelephoraceae	Taylor and McCormick (2008)
ITS86F/ITS4	ITS86F (F) ITS4 (R)	GTGAATCATCGAATCTTTGAA TCCTCCGCTTATTGATATGC	ITS-2	59	Both Ascomycota and Basidiomycota (including some Tulasnella)	White *et al*. (1990)
ITS3/ITS4OF	ITS3 (F) ITS4OF (R)	GCATCGATGAAGAACGCAGC GTTACTAGGGGAATCCTTGTT	ITS-2	62	All Basidomycota	White *et al*. (1990) Taylor and McCormick (2008)
5.8S-Tulngs/ITS4-Tul2	5.8S-Tulngs ITS4-Tul2	CATTCGATGAAGACCGTTGC TTCTTTTCCTCCGCTGAWTA	ITS-2	57	All Basidiomycota (inc. Serendipitaceae and Tulasnellaceae)	Rammitsu *et al.* (2021) Oja *et al*. (2014)
NS7/ITS1OF-RC	NS7 (F) ITS1OF-RC-G (R) ITS1OF-RC-A (R)	GAGGCAATAACAGGTCTGTGATGC ACTTCCTCTAAATGGCCGAGTT ACTTCCTCTAAATGACCAAGTT	SSU	62	Some Ascomycota and Basidiomycota (including some *Tulasnella*)	White *et al*. (1990) Waud *et al*. (2014) Waud *et al*. (2014)
ITS1OF/ITS2 m	ITS1-OF (F) ITS1-OF (F) ITS 2 m	AACTCGGCCATTTAGAGGAAGT AACTTGGTCATTTAGAGGAAGT TCGCTGCGTTCTTCATCGA	ITS-1	62	Some Ascomycota and Basidiomycota (including some *Tulasnella*)	Taylor and McCormick (2008)
ITS1F/ITS2	ITS1F (F) ITS2 (R)	CTTGGTCATTTAGAGGAAGTAA GCTGCGTTCTTCATCGATGC	ITS-1	62	Both ascomycota and basidomycota (including some Tulasnella)	Gardes and Bruns (1993) White *et al*. (1990)
ITS4OF-RC/cNL2F	ITS4OF-RC (F) cNL2F (R)	AACAAGGATTCCCCTAGTAAC GTTTCCCTTTTAACAATTTCAC	LSU	59	Some Ascomycota and Basidiomycota (including some Tulasnella)	Waud *et al*. (2014) White *et al*. (1990)

Table 5 - List of endophytic fungi that has been isolated and identified from root by using a broad-spectrum primer (ITS1 and ITS4)

Orchid Species	Endophytic fungal (Accession no./taxonomic affiliation)	Type of primer	Country	References
Vanda wightii	*Ceratobasidium_Wyd1* (MW59578)	ITS1 and ITS4	India	Suresh *et al.* (2023)
Dendrobium longicornu	*Alternaria* sp. (MN256650), *Cladosporium* sp. (MN256649), *Coniochaeta* sp. (MK225602), *Penicillium* sp. (MN256653), *Fusarium* sp. (MN256645), *Fusarium* sp. (MN256647), *Fusarium* sp. (MN256646).	ITS1 and ITS4	Nepal	Shah *et al*. (2022)
Aerides rosea	*Tulasnellaceae* sp. (JF691200)	ITS1 and ITS4	China	Zhao *et al. (*2021)
Dendrobium nobile	*Tulasnella deliquescens (*LC175331)	ITS1 and ITS4	China	Zhao *et al*. (2021)
Dendrobium cucullatum	*Tulasnella* sp. strain SSCDO-4 (MH348613	ITS1 and ITS4	China	Zhao *et al*. (2021)
Epigeneium amplum	*Tulasnella* sp. 140 (AY373281)	ITS1 and ITS4	China	Zhao *et al.* (2021)
Gastrochilus calceolaris	*Ceratobasidium* sp. GC (GQ369961), *Ceratobasidium* sp. FPUB 168 (EF536969), *Rhizoctonia* sp. Abn1b (AJ318432), *Rhizoctonia* sp. Onv6 (AJ318436)	ITS1 and ITS4	Bangladesh	Hossain (2019)
Aerides multiflora	*Ceratobasidum* sp. (JX913820), *Ceratobasidum* sp. (JX913820),	ITS1 and ITS4	India	Bhatti *et al.* (2017)
Paphiopedilum villosum (Lindl.) Stein.	*Tulasnella* sp. (AY373281)/Tulasnellaceae *Rigidoporus vinctus* (HQ400710)/ Polyporales *Ceratobasidium* sp. (HM117643)/Tulasnellaceae *Flavodon flavus* (JQ638521)/Polyporales *Nigroporus vinosus* (AB811859)/Polyporales *Coriolopsis retropicta* (KC867403)/Polyporales *Valsa eugeniae* (AY347344)/Diaporthales	ITS1 and ITS4	Thailand	Khamchatra *et al*. (2016)
Aerides multiflorum	*Ceratobasidium* sp. (Eu605733)	ITS1 and ITS4	western Himalayas	Hossain *et al.* (2013)
Rhynchostylis retusa	*Ceratobasidium* sp. (Eu605732)	ITS1 and ITS4	western Himalayas	Hossain *et al*. (2013)
Pecteilis susannae (L.)	*Epulorhiza* sp. GQ856216 *Epulorhiza* sp. GQ856215 *Epulorhiza* sp. GQ856214 *Fusarium* sp. GQ862347 *Epulorhiza* sp. FJ882028 *Epulorhiza* sp. GQ862346 *Epulorhiza* sp. FJ940903 *Epulorhiza* sp. FJ873174	ITS1 and ITS4	Thailand	Chutima *et al.* (2011)

(Taylor and McCormick 2008). To address this issue, the ITS4-Tul primer has been designed to study only *Tulasnella* species, thereby minimising the amplification of other taxa. Two primers that are *Tulasnellla* specific which is ITS4-Tul and ITS4R are designed from the 3-end of ITS2 (Suárez *et al.*, 2006). The ITS4-Tul primer is a perfect or near-perfect match for some of the core species of Tulasnella but their mismatches with the majority of other fungi make them a specific primer. ITS4-Tul has been used widely as a primer, especially for the identification of orchid mycorrhizal primarily targeted Tulasnellaceae, which are mostly reported to have the ability to promote seed germination (Oja *et al.*, 2014; McCormick *et al.*, 2021; Suetsugu *et al.*, 2021). Meanwhile, ITS1-OF and ITS4-OF is nowadays are increasingly used in characterising orchid fungal symbionts as they are designed to be a broad spectrum basidiomycete specific primer (Currah and Sherburne, 1992; Taylor and McCormick, 2008; Jacquemyn *et al.*, 2010).

A study on identification of fungi identification of terrestrial orchid mycorrhizal by using broad spectrum fungal taxa primer (ITS86F/ITS4) by Waud *et al*. (2014) has outperformed the other primer pair. The study also assessed the efficacy of several type of broad-spectrum primer and specific primer for orchid mycorrhizal fungi to understand and characterized orchid mycorrhizal communities and suggested several suitable primer pairs. Other study also uses the broad-spectrum primer pair ITS86F/ITS4 to investigate the orchid mycorrhizal community in both epiphytic and terrestrial orchid (Cevallos *et al*., 2017; Johnson *et al*., 2021). However, the use of broad-spectrum primer for identification of orchid mycorrhizal fungi is constrained by a primer bias, which arise from the inability of the primer to identify a specific fungus within a sample due to the mismatch during PCR. While Tulasnellaceae fungi are commonly associated with orchids (Dearnaley *et al*. 2012), their molecular detection poses challenges due to mismatches with universal fungal primers (Suárez *et al*., 2006; Taylor and McCormick 2008; Waud *et al*., 2014; Rammitsu *et al*., 2021).

Moreover, previous comprehensive investigations conducted through Sanger sequencing-based methodologies have indicated distinctions between the mycorrhizal communities associated with epiphytic orchids and those associated with terrestrial orchids (Martos *et al.,* 2012; Xing *et al.,* 2019). The utilising of Tulasnellaceae-specific primers for the assessmen of orchid mycorrhiza; networks by metabarcoding analysis is highly recommended, particurlay in the context of epiphytic orchids, as emphasised in the research conducted by Rammitsu *et al*. (2021). The commonly used broad spectrum primer, ITS86F/ITS4 effectively identified *Ceratobasidiaceae* and *Serendipitaceae* fungi but proved inadequate in detecting the diversity of *Tulasnellaceae* fungi (Rammitsu *et al.,* 2021). Due to significant primer biases present within the Tulasnellaceae family, which plays a crucial role as mycorrhizal symbionts in the majority of orchid species, it is imperative to exercise caution in selecting primers and thoroughly assess potential biases (Oja *et al*., 2014).

8. Sequencing

When it comes to fungi, morphology is often the method of choice for performing the fundamental function of species distinction. However, distinguishing species based on their morphology can be difficult, particularly for fungi that do not have complex fruiting bodies, as is the case with the three families of Rhizoctonia species that are linked with orchids (Gardes and Bruns, 1993). Conventionally, it has been thought that the 'Rhizoctonia' complex, which includes species from three different fungal families (Tulasnellaceae, Ceratobasidiaceae, and Serendipitaceae), makes up the bulk, if not the entirety, of orchid mycorrhizal fungus. However, recent research suggests that this may not be the case. Septal ultrastructure is a defining characteristic that separates the various clades within Rhizoctonia (Currah and Sherburne, 1992), but careful inspection is still required to distinguish Sebacinaceae and Tulasnellaceae (Andersen, 1996). This problem is compounded by the fact that when the cryptic, resupinate fruiting structures are seldom observed. Basidial morphology offers suitable identification of orchid-associated Rhizoctonia species at the morphospecies level (Warcup and Talbot, 1967). However, orchid isolates are rarely induced to fruit in culture as some fungi cannot be produced in artificial circumstances (Currah *et al*., 1990).

In order to expand knowledge of fungal variety, culture-independent technologies (sequencing and cloning) have been created. Morphological identification methods are conventional identification method that involves evaluating the

morphological and microscopic features of fungi on different culture media and under different conditions. This method can be accompanied by other identification methods to help identify fungi more accurately. Other methods, such as microscopic examination or biochemical screening, can be performed alone or in conjunction with molecular analysis. With the recent development of advanced molecular techniques (e.g., next-generation sequencing), the spectrum of fungi discovered at the species level has expanded significantly, allowing for more precise ecological inferences (Peay, 2014).

High-Throughput Sequencing (HTS) technologies provide a number of benefits, including the capability to identify fungi at trace levels, quick microbial community structure analysis, and cost-effectiveness (Cruz *et al.,* 2014; Tedersoo and Nilsson, 2016; Nilsson *et al.,* 2019). These benefits can be found in HTS technologies. According to Nilsson *et al*. (2019), a typical HTS metabarcoding process consists of several important stages, including DNA extraction, marker-based PCR amplification, DNA sequencing, sequence processing, and data analysis. These processes are listed in the order as follows: sampling then DNA extraction. However, one potential downside of these technologies is that they may potentially result in the spread of pollutants and mycorrhizal fungi that are not specific to orchids.

Research methodology and sequencing carried out on high-throughput platforms are the two components of the most typical approaches to molecular identification. DNA microarrays, clone libraries, denaturing gradient gel electrophoresis, fluorescence *in situ* hybridization, and gene chip approaches are some of the other methods that can be utilised for the identification of fungi (Dearnaley, 2007). However, these technologies have shortcomings such as limited throughput, time-consuming processes, and lower accuracy. Additionally, they have been overshadowed by the growing popularity of alternative methods such as the MiSeq PE300 and HiSeq PE250 platforms (Julou *et al*., 2005).

Furthermore, alternative methods, such as using an Illumina NovaSeq/HiSeq sequencer and the application of shotgun metagenomic technology, provide access to functional gene information from all microorganisms within a community through genomic DNA analysis (Bahram *et al.,* 2018; Fadiji and Babalola, 2020). These methods were developed by Bahram *et al*. (2018) and Fadiji and Babalola (2020). An important step forward in orchid mycorrhiza research has been taken thanks to the development of this technique and the growing availability of orchid and reference orchid mycorrhizal fungal genomes (Zhang *et al*., 2016).

Because of their ability to simultaneously sequence a mixed DNA template across numerous samples with a high sequencing depth (Nilsson *et al*., 2019), next-generation sequencing (NGS) approaches have become practically widespread in mycorrhizal research in recent years. This is partly owing to the fact that NGS methods have grown more affordable in recent years. In contrast, sequencing DNA from individual mycorrhizal root tips may be ideal for Sanger sequencing when it comes to detecting shifts in regularly occurring fungus species (Shemesh *et al*., 2020). This was found by Shemesh and colleagues. In contrast to next-generation sequencing technologies, which can process millions of DNA fragments simultaneously, the Sanger sequencing method only processes one DNA fragment at a time (Slatko *et al*., 2018). This makes the Sanger sequencing method superior in terms of sequencing volume. This distinction has the ability to bring forth different conclusions regarding the make-up of the community.

9. Conclusions

This review provides an overview of the most significant literature in orchid mycorrhizal fungi from about 2002-2023. The molecular identification of orchid mycorrhiza represents a significant advancement in our understanding of the complex relationships between orchids and their mycorrhizal fungal. In addition, finding the most appropriate extraction method and choosing a suitable primer for amplification is essential to ensure accurate identification. Moreover, the utilization of molecular techniques compliments morphology-based identifications offers a reliable, unbiased, and frequently more precise tools for confirming species. It is particularly beneficial for cryptic species, hybrids, morphological variables organism such as mycorrhizal, or situations when usual identification methods fail. Based on the review, the ITS regions prove to be a great primer in the field of mycorrhizal studies to its inherent variability, widespread applicability and straightforward amplification process and compatibility with established databases. This technique enables researchers to accurately identify the specific fungal species

associated with a particular orchid species and to investigate the functional tole of these fungi in orchid growth and development. With the advancement of molecular techniques, it is now possible to examine the genetic diversity of these fungi and understand the evolutionary relationship between different orchid mycorrhizal fungi. These may lead to the development of new conservation strategies for these unique and valuable plant species.

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