



Original Research

Optimising Sucrose Concentrations for Efficient *In Vitro* Propagation of Citrus Rootstocks

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ABSTRACT

Citrus is one of the most key members of the Rutaceae family and a widely grown fruit crop worldwide, containing considerable amounts of vitamin C and flavonoids. Rootstocks were used because they are resistant to soil-borne diseases and promote healthy production. Seeds were taken from citrus rootstocks and cultured in MS media with different concentrations of sucrose, M1 (30 g L⁻¹ sucrose) and M2 (15 g L⁻¹). In this study, eight rootstocks were collected for in vitro regeneration: citrumelo (*Citroncirus spp.*), citron (*Citrus medica*), Volkamariana (*Citrus volkamariana*), Rangpur lime (*Citrus limonia*), rough lemon (*Citrus jambhiri Lush*), sour orange (*Citrus aurantium*), Kharna Khatta (*Citrus Kharna*) and Carrizo citrange (*Citrus sinensis*). These materials were used for further propagation and regeneration through tissue culture. All these rootstocks were capable of regenerating and producing in vitro plantlet shoots. Data were collected on days to initiate germination, contamination, number of leaves per plant, leaf length (mm), leaf width (mm), leaf area (mm), and plant height (mm). The data were analysed using STATISTIX 8.1, and the means were compared using the least significant difference (LSD) test at the 5% significance level. The maximum leaf area (203.55 cm²) and maximum plant height (19.07 cm) were observed in citrange grown in MS media 1 (M1). The maximum leaf number (13.77) was observed in sour orange in MS media 1 (M1). Regenerated plantlets were transplanted for acclimatisation to obtain the best results. Therefore, a maximum of 96.6% plantlets of citrange and a minimum of 86.6% plantlets of Kharna Khatta were successfully shifted from MS media 1 containing 30 g L⁻¹ sucrose. However, a maximum of 90% of the plantlets of Kharna Khatta and a minimum of 10% of Citrumelo were successfully shifted from MS media 2 containing 15 g L⁻¹ sucrose.

Keywords: Sucrose concentrations, citrus rootstocks, propagation, regeneration, micropropagation.

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INTRODUCTION

Owing to its rapid development and abundant supply, citrus is considered one of the most important fruits in the world. Citrus, a vital fruit crop grown worldwide, is a member of the Rutaceae family. Citrus is well-known for its delicious flavour, pleasant scent, and attractive appearance. It is also very nutrient-dense. In addition to having 3-4% sugar and essential minerals such as calcium and magnesium, Citrus is an excellent source of vitamin C. Citrus is currently grown in tropical and subtropical climes worldwide, despite being thought to have originated in Southeast Asia, specifically in places such as China and Indonesia

(Wang and Zhang, 2023). Citrus is the second most produced and cultivated crop in the world, after grapes (Pérez and García, 2024). It is estimated that 7.13 million tons of citrus are produced annually worldwide. Pakistan is ranked 12th among countries that produce mandarin. Mandarins make up approximately 70% of all citrus production, with Kinnow mandarins accounting for more than 95% of Pakistan's citrus production (Cheema and Jamali, 2020). With 95% of the country's citrus production coming from Punjab, Sargodha alone is responsible for 47.9% of the country's Kinnow production. Toba Tek Singh, Vehari, Khanewal, and Multan are among the other districts that make a substantial contribution (Kumar and Singh, 2023). Pakistan exports approximately 250,000 tonnes of Kinnow Mandarins each year to countries such as Southeast Asia, the Middle East, and Russia, where they are mostly consumed fresh and generate approximately \$120 million in foreign cash (Raza and Ahmed, 2016). Citrus fruits, including oranges, mandarins, lemons, limes, and grapefruits, grow differently in different climates, which affects their quality (Fernández and López, 2023). Fruit quality can be adversely affected by biotic factors (such as microbes, insect pests, and

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diseases) and abiotic factors (such as wind, fruit splitting, sunburn, and nutritional imbalances). Oil coatings are frequently used to control pests and diseases, and postharvest treatments are commonly applied to perishable citrus fruits to ensure food safety (Vanaclocha et al., 2019). Despite its significance, Pakistan's average citrus production is only 9-10 tons per hectare, whereas other citrus-producing nations produce 26 tonnes per hectare (Sharma and Singh, 2009). This inconsistency underscores the importance of effective nutrition management to increase citrus output and growth. Citrus is commercially grown asexually by grafting or budding to ensure consistent quality and higher yields. On the other hand, rootstocks are usually sexually reproduced through seeds (Kumar and Shekhawat, 2024). Fruit granulation, acid concentration, juice content, and exterior traits, including size, shape, and colour, are all influenced by rootstocks, which are crucial in determining fruit quality. For example, research comparing the rootstocks of rough lemon (*Citrus jambhiri* Lush.) and sour orange (*Citrus aurantium* L.) has shown notable variations in fruit quality (Li and Chen, 2024). Because seed-grown plants take 7-12 years to develop fruit and are susceptible to soil-borne diseases and pests, including aphids, citrus leaf miners, citrus whiteflies, root rot, and citrus canker, the use of rootstocks is beneficial, as citrus is also susceptible to cold. Conversely, grafted plants exhibit increased resistance to infections, such as *Pseudomonas syringae* and *Xanthomonas axonopodis*, as well as viruses like citrus tristeza virus, and they begin to bear fruit within 3 to 4 years. The scion becomes more resistant to environmental stressors and soil-related problems due to the rootstock's improved water and nutrient uptake, greater photosynthetic storage, and enhanced hormone synthesis. The present study aims to explore the regeneration of new rootstocks resistant to soil-borne diseases and their healthy production through in vitro regeneration via tissue culture techniques, generating virus-free plantlets from existing rootstocks and producing a healthier nursery of seedlings. In tissue culture regeneration, exact copies of plants with desirable traits are produced in a short time, requiring less space.

MATERIALS AND METHODS

The experiment was conducted in the tissue culture lab of the Department of Horticulture at the University of Layyah.

Collection of rootstocks

Initially, rootstock fruits were gathered from the University of Agriculture, Faisalabad, and the Citrus Research Station in Sargodha. For further propagation, seven rootstocks were utilised: Citrumelo (*X Citroncirus* spp.), citron (*Citrus medica*), Volkamariana (*Citrus volkamariana*), Rangpur lime (*Citrus limonia*), rough lemon (*Citrus jambhiri* Lush), sour orange (*Citrus aurantium*), Kharna Khatta (*Citrus Kharna*), and Carrizo citrange (*Citrus sinensis*).

Extraction and storage of seeds

The fruit seeds were removed, and the fruit pulp was removed by washing them with tap water, then rinsing with two to three drops of Tween 20 detergent. After being cleaned with distilled water, the seeds were treated with a fungicide and allowed to

Table 1: Concentrations of two media used for in vitro regeneration of citrus rootstocks.

Ingredients	M ₁ L ⁻¹	M ₂ L ⁻¹
Macro nutrients	100 mL	100 mL
Micronutrients	1 mL	1 mL
Iron	10 mL	10 mL
Vitamin	10 mL	10 mL
Sucrose	30 g	15 g
Agar	8 g	8 g

dry in the shade. For future propagation, the seeds of every rootstock were labelled and kept in a refrigerator at 4 °C.

Sterilization

Autoclaving was used to disinfect the glassware, equipment, and culture media. The process of autoclaving involves using water vapour for at least 20 minutes at 121 °C (250 °F) and 15 psi. After the transplants or seeds are sterilised with tap water to eliminate the fungicide, they are rinsed with two to three drops of Tween 20 detergent, steeped for three to four minutes in a 3% sodium hypochlorite solution, and then rinsed three times with double-distilled water.

Murashige and Skoog media concentration

To prepare the media, a magnetic stirrer was used to thoroughly combine all these nutrient components (Murashige and Skoog, 1962). To test the impact on germination, two media were prepared with identical nutrient compositions: media 1 (M1) and 2 (M2), except for sucrose, which was present at 30 g L⁻¹ in media 1 and 15 g L⁻¹ in media 2 (Table 1).

Hardening and acclimatisation

After being removed from the test tube, the successfully regenerated plantlets were cleaned with double-distilled water to remove the media. They were then placed in plastic pots filled with autoclaved sand supplemented with fungicide and NPK and covered with a polythene bag to maintain stable humidity levels. After 15 days, the polythene bags were removed, and the plantlets were transplanted into plastic pots filled with autoclaved soil for rooting, then placed in a greenhouse for 1



Figure 1: Stages of hardening A: seed germination, B: seedlings, C: plantlet, D: maturation, E: autoclaved sand, F: pots, G: plantlets transferred to sand covered with a polythene sheet, H: acclimatization.

month (Fig. 1).

Data collection

The following data were collected.

Germination percentage

The following formula was used to determine the germination percentage of regenerated plants:

Germination (%) = Total number of germinated seeds / total number of cultured seeds × 100

Contamination percentage

The following formula was used to determine the contamination percentage of regenerated plants:

Contamination percentage (%) = Total number of contamination / total number of cultured seeds × 100

Number of leaves per plant

After a month of seeding in a test tube, the total number of leaves per plant was visually counted.

Length of leaves

Each plant's three leaves were measured for length using a digital vernier calliper (DC-154A).

Width of leaves

The width of three leaves was measured using a digital vernier calliper (DC-154A).

Leaf area

The following formula was used to determine the leaf area:
Leaf area = length (mm) × width (mm) × correction factor (0.75)

Plant height

The following formula was used to obtain the leaf area: a digital vernier calliper (150 mm) was used to measure height of each plant.

Statistical analysis

This experiment was conducted in a CRD design with three replicates, each with 10 test tubes. STATISTIX 8.1 Software was used to analyse the data, and the least significant difference (LSD) test was used to compare the means at the 5% probability level.

RESULTS AND DISCUSSION

Leaf area

The direct regeneration approach was used to regenerate the shoots of the citrus rootstock. *In vitro*, citrus plant growth and

quality were observed at various sugar concentrations. Direct regeneration was used in this citrus rootstock shoot regeneration study to assess how various sucrose concentrations affect plant growth *in vitro* (Fig. 2). The findings

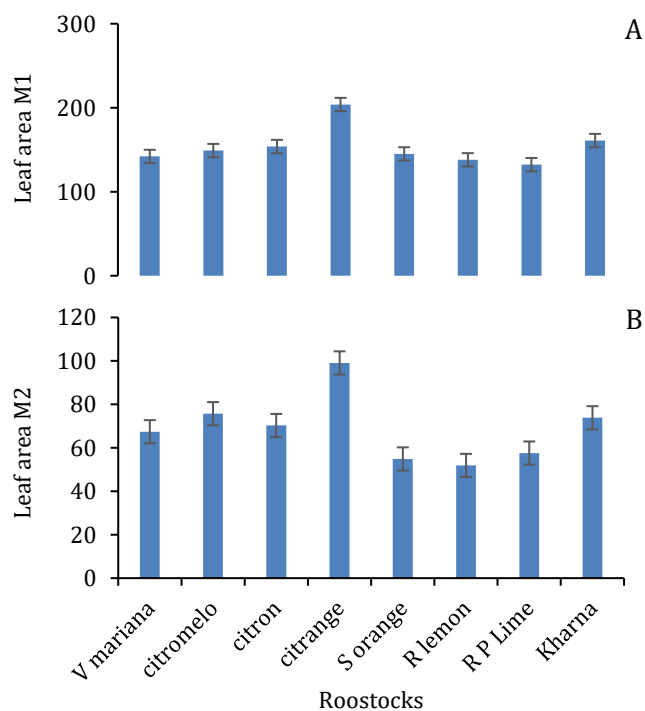


Figure 2: Comparison of the leaf areas of the rootstocks of citrus plants in MS media 1 (A) and MS media 2 (B).

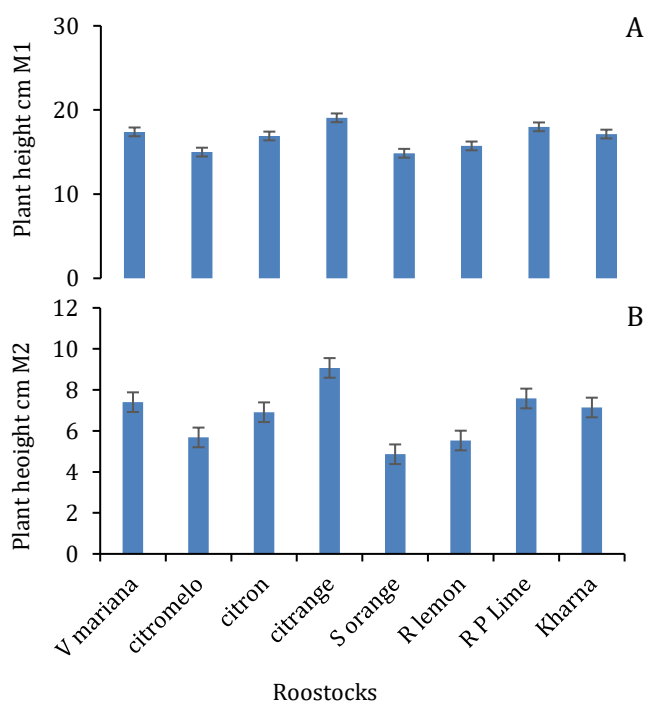


Figure 3: Comparison of the effect of MS media 1 (A) and MS media 2 (B) on height of citrus plant.

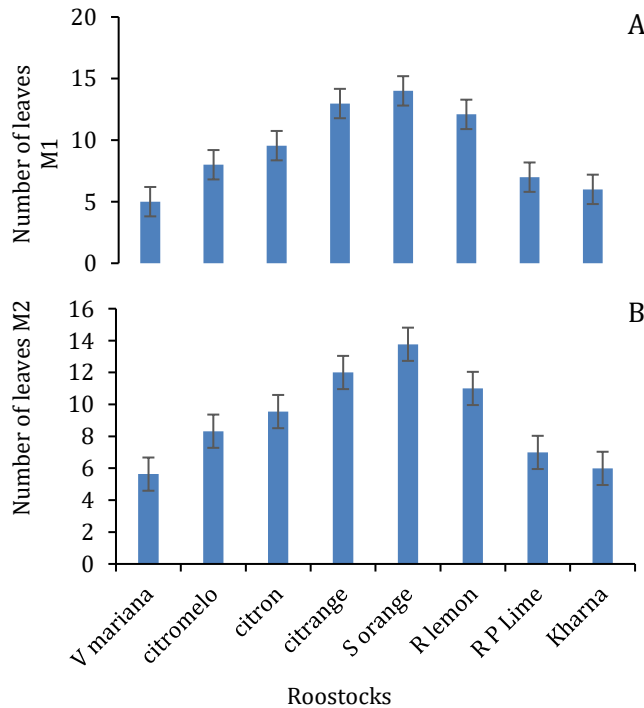


Figure 4: Comparison of the effect of MS media 1 (A) and MS media 2 (B) on citrus plants in terms of leaf number.

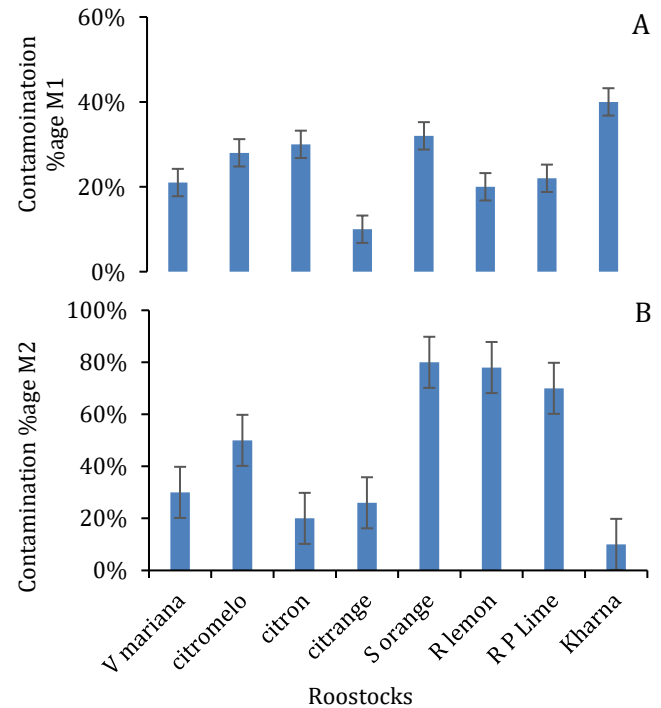


Figure 6: Contamination percentage of seeds with 3 replications on basal MS medium 1 (A) and MS media 2 (B).

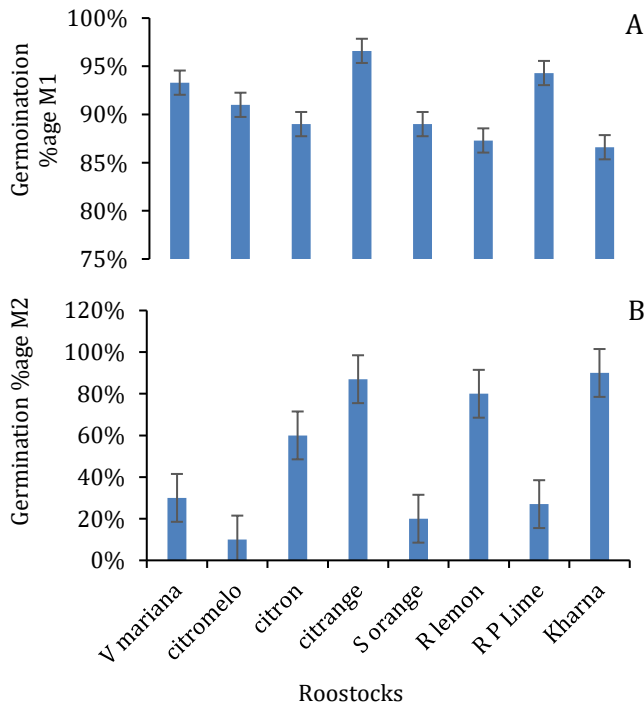


Figure 5: Germination percentage of seeds with 3 replications on basal MS media 1 (A) and MS media 2 (B).

demonstrated that, in comparison with lower sucrose levels, a 30 g L⁻¹ sucrose concentration in MS medium 1 markedly increased plant growth (Fig. 2). Previous studies have shown that sucrose is essential for promoting plant development in

tissue culture (Aleza et al., 2009) and is often necessary for optimal growth. Secondary metabolism in tissue culture is also affected. Under 30 g L⁻¹ sucrose in MS medium 1, the leaf area significantly increased in citrange (203.55) and decreased in the rough lemon (135.95) (Fig. 2). However, both types of leaf area decreased at a lower sucrose content of 15 g L⁻¹ in MS medium 2, with citrange displaying 99.07 g L⁻¹ and rough lemon showing only 51.87 g L⁻¹. A thorough examination of the physical and behavioural characteristics of the leaf stomata and transpiration rates in comparison with those of diploid vs triploid citrus plants revealed that the leaf area of diploid *Citrus clementina* was 28.5±9.5 (George and Debergh, 2008).

Shoot height

Citrange plants grew taller (19.07 cm), and sour orange plants grew shorter (14.86 cm) in this experiment when cultivated in MS media 1 with 30 g L⁻¹ sucrose. On the other hand, citrange's height increased less (9.07 cm) and sour oranges reduced even more (4.86 cm) when 15 g L⁻¹ sucrose was added to MS medium 2 (Fig. 3). Plant height was noted in citrange (5.7), bittersweet orange (10.4), and sour orange (9.3). Furthermore, growth regulators significantly affected shoot formation; explants from shoot tips had a higher shoot-forming percentage than those from nodal segments. The maximum shoot development (84%) was obtained from shoot tips cultivated in MS media supplemented with 1.0 mg L⁻¹ growth regulators (Romero and García, 2023).

Number of leaves

Citrus roostocks showed significantly different leaf counts in MS media 1 containing 30 g L⁻¹ sucrose. Sour orange had the

most leaves (13.77), whereas Volkamariana had the fewest (5.63) (Fig. 4). Similarly, the number of citrange leaves decreased (2.97), whereas the number of rough lemons increased (6.00) in MS medium 2 supplemented with 15 g L⁻¹ sucrose (Fig. 4). These results demonstrate how the concentration of sugar affects the growth of leaves. Both sucrose levels and growth regulator treatments are essential for leaf formation in citrus tissue culture, as evidenced by the average of 5.41 leaves during shoot regeneration in *Citrus hystrix* under varying concentrations of plant growth regulators after eight weeks (George and Debergh, 2008).

Germination percentage

Citrange had the highest germination rate in this experiment (96.6%). In contrast, Kharna Khatta had the lowest germination rate (86.6%) (Fig. 5). When cultivated in MS media 1 with 30 g L⁻¹ sucrose, citrange exhibited a higher germination rate and a low contamination rate of 10%. In contrast, Kharna Khatta presented a greater contamination rate of 40%. Kharna Khatta exhibited better germination (90%) in MS medium 2 with 15 g L⁻¹ sucrose, whereas the percentage of citrumelo decreased to 10%. A prior study that assessed the effects of medium and surface sterilisation on *Citrus sinensis* micro cutting revealed that 87.7% of the seeds germinated (Vanaclocha et al., 2019).

Contamination percentage

According to this study, citrange had the lowest contamination rate at 10%, while Kharna Khatta had the lowest at 40% (Fig. 6). Citrange also had the highest germination rate (96.6%), indicating a notable improvement. Still, Kharna Khatta had a lower rate (86.6%). Moreover, citrange had the lowest contamination percentage (10%) and the highest germination in MS media 1 supplemented with 30 g L⁻¹ sucrose, whereas Kharna Khatta had the highest contamination (40%) in the same medium. Kharna Khatta demonstrated increased germination of up to 90% in MS medium 2 supplemented with 15 g L⁻¹ sucrose, but the percentage of citrumelo markedly decreased to 10%. In this media state, contamination rates were incredibly low in Kharna Khatta (10%) but increased dramatically in citrange when it was grown with rough lemon and Rangpur lime (up to 80%). In contrast, Vestri et al. (2003) evaluated the effects of surface sterilisation and various growth conditions on in vitro micro cuttings of *Citrus sinensis*, reporting a germination rate of 87.7% and a contamination rate of 13.3%. These findings demonstrate how different citrus rootstocks respond differently to media composition and sucrose concentration, with respect to contamination and germination. The lowest contamination of 86.6% in citrange (10%), and the highest contamination of 40% in Kharna Khatta (Fig. 6). According to this experiment, the percentage of germination increased by 96.6% in citrange. In contrast, it decreased by 86.6% in Kharna Khatta. In contrast, the rate of contamination was increased by 40% in Kharna Khatta and decreased by 86.6% in citrange 10% in MS media 1 with 30 g L⁻¹ sucrose (Altman et al., 1990).

CONCLUSION

In vitro rootstock-reproduction methods are required to generate true-to-type, disease-free planting material. Citrumelo (*X Citroncirus* spp.), citron (*Citrus medica*), Volkamariana (*Citrus*

Volkamariana), Rangpur lime (*Citrus limonia*), rough lemon (*Citrus jambhiri* Lush), sour orange (*Citrus aurantium*), Kharna Khatta (*Citrus Kharna*), and Carrizo citrange (*Citrus sinensis*) are the eight citrus rootstocks cultivated in vitro in two types of MS media with varying concentrations of sucrose (30 g L⁻¹ in media 1 and 15 g L⁻¹). The maximum leaf area, plant height, number of leaves, germination rate, and contamination rate were examined in media 2. The optimal way to transplant hardened in vitro plantlets is into autoclaved soil. Citrange had the highest survival rate in MS media 1 at 30 g L⁻¹. Of the two media types, Media 1 performed best.

DECLARATION OF COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Umbreen Shahzad: Supervision, Project administration, review and editing, **Momal Batool:** Data collection, writing, original draft resources, data curation, software, editing, methodology, **Tahira Abbas:** Conceptualisation, investigation, formal analysis, **Muhammad Ammar Raza:** Conceptualisation, investigation, data correction, review & editing.

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ETHICAL STATEMENT

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

All data is published in this research article.

INFORMED CONSENT

Not applicable.

CONSENT FOR PUBLICATION

All authors consented to the publication of this research article in the Journal of Horticulture Sciences and Technology.

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