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Influence of Plant Growth regulators and pH on *Chaenomeles japonica* L. Micrograft Survival and growth

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Abstract

Chaenomeles japonica L. exhibits optimal growth in soils with neutral to slightly acidic pH, whereas alkaline soils, as a result of limited iron absorption and induce leaf chlorosis. *In vitro* micrografting represents a key technique for the micropropagation of diverse plant species. Micrografting of *C. japonica* onto tolerant rootstocks could be an effective strategy to enhance iron uptake efficiency. The major objectives of this study were to identify the most appropriate culture medium, and assess the responses of successfully micrografted plants to varying culture medium pH levels. The culture medium employed consisted of half-strength MS consisted of different plant growth regulators. The grafting method employed was a cleft micrografting of *C. japonica* onto *P. coccinea* rootstock. Micrografted plants that exhibited successful graft in the previous experiment were transferred to culture media with different pH levels (5.7, 5.9, and 6.1). The grafting success in micrografting of *C. japonica* onto *P. coccinea* rootstock is influenced by both media. In several instances, non-injected treatments resulted in the highest scion growth. The most effective condition was the injected medium supplemented with either 2 mg L^{-1} BAP or 1 mg L^{-1} IBA. The micrografting process improved the uptake of iron and manganese, suggesting a functional modification in the nutrient acquisition pathways during transport from the root system to the scion. These results suggest that utilizing the *P. coccinea* rootstock for micrografting *C. japonica* could be a viable strategy to mitigate iron chlorosis, a common affliction in alkaline soils that reduces chlorophyll content.

Keywords: Iron, Leaf chlorosis, Micrografting, ornamental shrub, pH media

Key Points

Hormonal protocols and delivery methods drive efficient micrograft organogenesis.

Optimal pH levels uniquely support graft-union development over basic nutrition.

Rootstock selection effectively overcomes iron deficiency in woody micrografts.

Introduction

Japanese quince (*Chaenomeles japonica* L.) is a widely utilized ornamental shrub in urban landscaping. This species thrives in neutral to slightly acidic soils; however, in alkaline or calcareous environments, limited iron availability often leads to leaf chlorosis and yellowing of young foliage during the growing season (Rumpunen 2002). Due to its low mobility within the plant, iron deficiency impairs chlorophyll synthesis, resulting in characteristic interveinal chlorosis (Singh 1988). In calcareous soils, iron deficiency is further manifested by reduced root dry weight, diminished shoot development, and significant alterations in plant iron concentrations (Mahmoudi et al. 2005; Tabatabaei et al. 2011). High bicarbonate concentrations in these soils elevate the pH of the soil solution, thereby interfering with nutrient uptake mechanisms and exacerbating chlorosis (Covarrubias and Rombolà 2013). While various mitigation strategies have been proposed—such as soil pH regulation, soil or foliar iron fertilization, trunk injection, and soil acidification—each approach possesses distinct limitations (Shamiri et al. 2017; Tagliavini and Rombolà 2001). Consequently, the use of iron chlorosis-resistant cultivars is recognized as one of the most economical and effective management strategies (Khoshgoftarmanesh et al. 2010). Since iron acquisition is genetically controlled, different cultivars exhibit varying responses to nutritional status even under identical environmental conditions (Clark 1990).

Plants are predominantly propagated by sexual and asexual means. Sexual propagation is associated with disadvantages including high heterozygosity, an extended juvenile phase, seed-borne diseases, loss of rootstock-related benefits, and reduced quality of the resulting production. Consequently, vegetative propagation techniques such as grafting, budding, cuttings, and layering are widely employed in horticultural crops (Tondihal and Jaryal 2023). *In vitro* micrografting represents a key technique for the micropropagation of diverse plant species, especially woody taxa. In recent decades, it has emerged as a valuable approach for promoting shoot regeneration and improving

the acclimatization of horticultural species grown under *in vitro* conditions (Wang et al. 2022). *In vitro* micrografting is performed in a humid, sterile environment under controlled conditions. *In vitro* cultures are stable, and the scion/rootstock are free of pathogens. Callus formation and rapid vascular reconnection between the scion and rootstock are crucial for successful graft union (Aazami and Hassanpouraghdam 2010; Ashrafzadeh 2020). Micrografting is a hybrid technique that integrates plant tissue culture with grafting technology, enabling the combination of tissue-cultured rootstocks with scions under *in vitro* conditions (Zhang et al. 2005). Compared to traditional grafting, *in vitro* micrografting is characterized by the use of smaller scions and rootstocks and the maintenance of sterile conditions throughout the procedure. Additionally, micrografting presents multiple advantages, such as year-round plant production, virus eradication, improved graft compatibility, and enhanced regeneration (Ashrafzadeh 2020). Because evaluating graft incompatibility in grafted trees may require months to years, micrografting serves as a valuable tool for early prediction of graft incompatibility during the initial growth phases of horticultural species (Darikova et al. 2011). The success of the micrografting technique relies on the establishment of a successful graft union between the rootstock and scion, and grafting proficiency is a critical determinant of success in *in vitro* grafting (Onay et al. 2004; Yıldırım et al. 2013). Micrografting success is influenced by the culture medium, plant species, and the source of explant material. Growth regulators in the culture medium may also impact grafting outcomes. Auxins and cytokinins are the most frequently used plant plant growth regulators in micrografting media (Zhu et al. 2007).

Iron deficiency chlorosis substantially impairs the aesthetic quality of *C. japonica* shrubs, particularly in urban landscapes. Accordingly, grafting C. *japonica* onto compatible and tolerant rootstocks can alleviate this disorder. *In vitro* grafting, owing to its noted advantages, may improve the performance of C. *japonica* under alkaline conditions and provides a model system to investigate iron uptake in rootstocks. Therefore, *in vitro* micrografting of C. *japonica* onto tolerant rootstocks could be an effective strategy to enhance iron uptake efficiency. Accordingly, the major objectives of this study were to identify the most appropriate culture medium and its application strategy, and assess the responses of successfully micrografted plants to varying culture medium pH levels.

Materials and Methods

Plant Materials

In this study, *P. coccinea* served as the rootstock, while *C. japonica* was used as the scion. Plant materials were sourced from plants grown in the landscape of Arak Municipality. All cuttings were collected from a single donor plant and propagated in the research greenhouse of Arak University under controlled environmental conditions (27 °C, 60% relative humidity, and 10,000 lux). Semi-hardwood cuttings were used for propagation. Cuttings, 15–20 cm in length, were collected in early spring and rooted in a moist perlite medium. After three months, the rooted plants were transplanted into pots filled with a 1:1 (v/v) mixture of agricultural soil and farmyard manure.

Surface Sterilization and Establishment of Explants

The healthy branches were removed from the central sections of the crown of plants cultivated in the greenhouse, ensuring that the *P. coccinea* branches (serving as rootstocks) measured 3-4 cm in length and possessed a minimum of two vegetative buds, while the C. *japonica* branches (acting as scions) were 1 cm long and contained one vegetative bud. The plant material underwent a thorough wash under running water with of Tween 20 (2–3 drops L^{-1}) for 30 min, followed by immersion in tobacco extract for 30 min (Taghizadeh and Aghili Gavari 2025). The explants were submerged in solution of 20% (v/v) home commercial bleach (Golrang®) containing NaOCl 5% for 17 minutes. Subsequently, explants were rinsed three times with sterile distilled water, then immersed in 80% (v/v) ethanol for 60 s, and finally washed three additional times with sterile distilled water. Under laminar airflow conditions, the ends of the explants were freshly trimmed to eliminate any damaged tissues that had been exposed to disinfectant agents, after which the micrografting procedure was initiated.

Culture Media and Culture Maintenance Conditions

The culture medium employed in this study consisted of half-strength Murashige and Skoog (MS) medium supplemented with different combinations of growth regulators (IBA, NAA, and BA). All treatments were supplemented with 15 g L^{-1} sucrose, 4 g L^{-1} activated charcoal (to inhibit phenolic exudation from explants into the medium), and 7 g L^{-1} agar. The medium pH was adjusted to 5.7–8.5 using 0.1 N HCl and NaOH. Following medium preparation, the vessels were sealed with aluminum foil and sterilized together with other required equipment in an autoclave at 121 °C and 1.2 atm for sterilization. After autoclaving, once the medium temperature cooled to approximately 45 °C, the selected antibiotic was aseptically added and thoroughly mixed into the medium. After culturing and micrografting, the explants were transferred to a growth chamber set at 19 ± 1 °C, under a light intensity of 4000 lux and a 16-h light/8-h dark photoperiod.

Evaluation of the Effects of Culture Medium and Rootstock on Micrografting Success

The rootstocks assessed in this experiment were *P. coccinea* and *C. japonica*. The culture medium (half-strength MS) and culture maintenance conditions were identical to those described previously. Following sterilization, micrografting was conducted under a laminar airflow cabinet. Explants measuring 3–4 cm in length with two vegetative buds were used for *P. coccinea* rootstocks, and 1 cm explants with a single vegetative bud were used for C. *japonica* scions. Rootstock and scion of similar diameter were selected for cleft grafting. A vertical incision of 3–5 mm was made on the rootstock using a scalpel. The scion was subsequently prepared with an oblique cut (V-shape) and inserted into the rootstock split, ensuring that the cambial layers of the rootstock and scion were fully aligned. Following the union of the rootstock and scion, 5 mL of sterilized culture medium, maintained at approximately 40 °C, was injected into the grafting union using a sterile syringe prior to solidification. In this experiment, the culture medium used to establish the micrografted plants was similar to the culture medium injected into the grafting union in terms of the type of plant growth regulator compound. The treatments consisted of hormone-free medium, 2 mg l^{-1} BA, 0.5 mg l^{-1} IBA, 1 mg l^{-1} IBA, 1 mg l^{-1} NAA, and 2 mg l^{-1} IBA. The establishment medium and the medium applied to the graft union contained identical growth regulator compositions, with the exception that the injected medium did not include antibiotics and agar. Three replicates of 3–4 cm explants of *C. japonica* and *P. coccinea*, without grafting and without medium injection, were included as controls. Following culture initiation, the plants subjected to graft-union injection were re-injected with sterilized culture medium three times at once every two weeks' intervals. The experiment was carried out as a factorial experiment in a completely randomized design (CRD) with three replications.

Evaluation of Rootstock and Culture Medium pH on the Growth of Micrografted Plants

Micrografted plants that exhibited successful graft unions in the previous experiment were transferred to culture media with different pH levels to assess their response to alkaline conditions. The culture medium employed in this experiment was similar to that used previously and consisted of half-strength MS supplemented with antibiotic, agar, activated charcoal, and plant growth regulators, but without sucrose because rooting had already been completed. The culture medium pH levels were set at 5.7, 5.9, and 6.1, and were adjusted prior to sterilization using 0.1 N NaOH. The plant materials evaluated comprised rooted explants of *C. japonica* rootstocks without grafting, micrografted C. *japonica* scions on C. *japonica* rootstocks, and micrografted C. *japonica* scions on *P. coccinea* rootstocks. The experiment was carried out as a factorial experiment in a completely randomized design with three replications.

Traits Measurement

From the first week of culture, visual traits including callus formation and micrograft success were assessed, and morpho-physiological characteristics were recorded eight weeks after micrografting. Callus formation was evaluated using a scoring system, with scores of 0, 1, 2, and 3 representing no callus, low callus, moderate callus, and high callus formation, respectively (Fig. 1).

Because the grafted plants could not be removed for trait measurements until the end of the experiment and the completion of rooting, growth-related traits were assessed using a scoring system with codes 1–5. Score 1 indicated the lowest scion growth, whereas score 5 indicated the greatest scion growth (Fig. 2). Greenness traits of both the rootstock and scion, including scion growth and visual parameters such as leaf color, vigor, and overall appearance of the rootstock and scion leaves, were assessed using a 1–5 scoring scale. Score 1 corresponded to the lowest level of greenness, and score 5 corresponded to the highest level of greenness. In micrografted plants, the number of adventitious roots emerging from each grafted plant was quantified and recorded. Roots with a minimum length of 1–2 mm were considered for this trait. After carefully removing the explants from the culture vessels, the roots were rinsed with distilled water to eliminate medium residues. Total plant length, scion length, rootstock length, and root length were subsequently measured (Fig. 3). Fresh and dry weights of the micrografted plants were determined following the evaluation of visual traits. The plants were placed in Petri dishes and oven-dried at 70 °C for 72 hours. Subsequently, the dry weight of the explants was measured and recorded using a digital scale with an accuracy of 0.001.

Total phenolic content in the samples was determined using the Folin–Ciocalteu colorimetric assay as described by Singleton and Rossi (1965). 400 µL of the diluted sample extract was combined with 2 mL of Folin–Ciocalteu reagent (1:10 dilution). Subsequently, 1.6 mL of 7.5% sodium carbonate solution was added to the mixture. The solution was then incubated in the dark at room temperature for 30 minutes. Following incubation, the absorbance of the samples was measured at 765 nm using a spectrophotometer. Total phenolic content was quantified using a standard curve generated from gallic acid, and results were expressed as mg of gallic acid equivalents/ g of dry weight

(mg GAE g^{-1} DW). Chlorophyll and carotenoid contents were determined according to Arnon (1949). 0.2 g of leaves from the middle part of the scions was ground in 80% acetone under dark conditions until the tissue was fully decolorized. The extract was adjusted to a final volume of 10 mL with 80% acetone in a graduated cylinder and centrifuged at 4000 rpm. Following centrifugation, the absorbance of the supernatant was recorded at 663 nm (chlorophyll a), 645 nm (chlorophyll b), and 470 nm (carotenoids) using a spectrophotometer. Subsequently, chlorophyll a, chlorophyll b, and carotenoid contents were calculated using the equations below and expressed as mg g^{-1} of fresh weight.

1- Chlorophyll a = (19.3*A663 - 0.86*A645) V/100W
2- Chlorophyll b = (19.3*A645 – 3.6*A663) V/100W
3- Carotenoides = 100 (A470) – 3.27(mg chl a) -104(mg chl. b) /227

V = denotes the volume of the supernatant obtained after centrifugation.
A = represents the absorbance measured at 663, 645, and 470 nm.
W= refers to the fresh weight of the sample (g).

The total fresh weight of each plantlet was measured using a digital scale with an accuracy of 0.001. The samples were placed in an oven at 70°C for 72 h, and their dry weight was measured. The samples were then powdered using a porcelain mortar and digested in 20% hypochloric acid (enough to cover the sample). Each sample was brought to a volume of 25 cc using distilled water (Brunori et al. 2004). After 24 h, the samples were filtered using filter paper, and the resulting solution was placed in a plasma emission spectrometer (ICP–OES, OPTIMA 3300 DV, Perkin-Elmer, USA) to determine plant elements, including Ca, Fe, Mg, Mn, K, Na, and Zn. First, the device was calibrated using a 23-element standard at concentrations of 0.5, 10, 15, 25, 50, and 100 ppm, and then a control sample (QC) with a concentration of 10 ppm was tested by the device.



a) Code 0 **b) Code 1** **c) Code 2** **d) Code 3**

Figure 1. Callus formation scoring at the graft union



a) Code 1 **b) Code 2** **c) Code 3** **d) Code 4** **e) Code 5**

Figure 2. Scoring system for *Chaenomeles japonica* scion growth



Figure 3. Measurement of root length in micrografted plants

Statistical Analysis

Data were analyzed using SAS software (version 9.1). Mean comparisons and statistical differences among treatments were assessed by Duncan's multiple range test (DMRT) at the 0.01 and 0.05 probability levels.

Results

Evaluation of the Effects of Culture Medium and Rootstock on Micrografting Success

The first signs of callus formation were observed 12 days after grafting in the C. *japonica* scion grafted onto *P. coccinea* rootstock without culture medium injection, cultured on half-strength MS medium without Plant growth regulator. The results indicated that although graft union formation occurred in most treatments and the scion grew on the rootstock, complete rooting and the development of a fully established grafted plant were achieved only in a limited number of the applied treatments. In none of the treatments involving self-grafting of C. *japonica* was root induction

or root growth observed, despite scion growth. This result indicates the difficulty of rooting in this rootstock and underscores the necessity of grafting onto a more easily rootable rootstock. Among the culture medium treatments, when C. *japonica* was grafted onto *P. coccinea* rootstock, the most effective treatment was the absence of culture medium injection at the graft union and the culture of micrografted explants on half-strength MS medium supplemented with 0.5 mg l^{-1} IBA (Fig. 4). In this treatment, in addition to sufficient rootstock and scion growth and callus formation, good rooting of the rootstock was also observed. In grafted plants on *P. coccinea* rootstock without culture medium injection at the graft union and with 2 mg l^{-1} BA, graft union formation and scion growth were observed; however, rooting occurred only to a limited extent (Table 1). The results obtained from this *in vitro* micrografting experiment of C. *japonica* onto *P. coccinea* rootstock demonstrate notable differences in rooting time under various hormonal and injection treatments. The earliest rooting occurred in explants treated with 2 mg l^{-1} BA followed by 1 mg l^{-1} IBA when the culture medium was injected into the graft union. Conversely, treatments without injection or with excessive IBA concentrations exhibited delayed root initiation (Fig. 5).

Table 1. The effect of rootstock and culture medium injection on micrografting success

Rootstock	Injection	Plant Growth regulator (PGR)	Scion growth (score)	Scion greenness (score)	Rootstock greenness (score)	Callus formation (%)	Number of roots
P. coccinea	With medium	1 mg L^{-1} IBA	3.00 cd†	3.00 f	3.33 e	1.33 e	1.33 b
		2 mg L^{-1} BA	2.33 e	2.00 i	2.33 h	0.00 h	0.66 c
		Free PGR	2.66 de	2.66 g	3.33 e	1.00 f	0.00 e
		0.5 mg L^{-1} IBA	3.00 cd	3.00 f	3.66 d	0.00 h	0.66 c
		1 mg L^{-1} NAA	3.33 bc	1.33 e	3.66 d	1.00 f	0.33 d
		2 mg L^{-1} IBA	4.33 a	4.33 b	4.66 b	2.00 c	0.00 e
	Without injecting	1 mg L^{-1} IBA	3.00 cd	2.00 i	3.33 e	2.00 c	0.00 e
		2 mg L^{-1} BA	4.66 a	4.66 a	4.33 c	2.33 b	0.33 d
		Free PGR	3.33 bc	1.33 e	3.33 e	1.33 e	0.00 e
		0.5 mg L^{-1} IBA	4.66 a	4.66 a	5.00 a	3.00 a	1.66 a
		1 mg L^{-1} NAA	4.66 a	4.66 a	4.66 b	2.00 c	0.00 e
		2 mg L^{-1} IBA	3.66 b	4.00 c	3.33 e	1.00 f	0.00 e
C. japonica	With medium	1 mg L^{-1} IBA	3.66 b	4.00 c	3.66 d	0.00 h	0.00 e
		2 mg L^{-1} BA	3.66 b	2.66 d	4.66 b	0.00 h	0.00 e
		Free PGR	1.33 f	2.33 h	3.33 e	1.33 e	0.00 e
		0.5 mg L^{-1} IBA	4.66 a	4.66 a	4.66 b	0.00 h	0.00 e
		1 mg L^{-1} NAA	4.66 a	4.66 a	4.66 b	0.00 h	0.00 e
		2 mg L^{-1} IBA	3.66 b	2.66 d	3.66 d	1.00 f	0.00 e
	Without injecting	1 mg L^{-1} IBA	3.66 b	2.66 d	3.66 d	0.66 g	0.00 e
		2 mg L^{-1} BA	2.66 de	2.66 g	3.66 d	1.66 d	0.00 e
		Free PGR	3.66 b	1.33 e	2.00 i	0.00 h	0.00 e
		0.5 mg l^{-1} IBA	2.66 de	2.66 g	2.66 g	0.00 h	0.00 e
		1 mg l^{-1} NAA	2.33 e	3.00 f	4.66 b	0.00 h	0.00 e
		2 mg l^{-1} IBA	4.66 a	4.66 a	4.66 b	1.66 d	0.00 e

†. Means within each column that share at least one common letter are not significantly different according to Duncan's multiple range test.



Figure 4. Rooted *Chaenomeles japonica* quince micrograft on *Pyracantha coccinea* rootstock (withouth medium injection) on half-strength MS + 0.5 mg l^{-1} IBA, six months after grafting

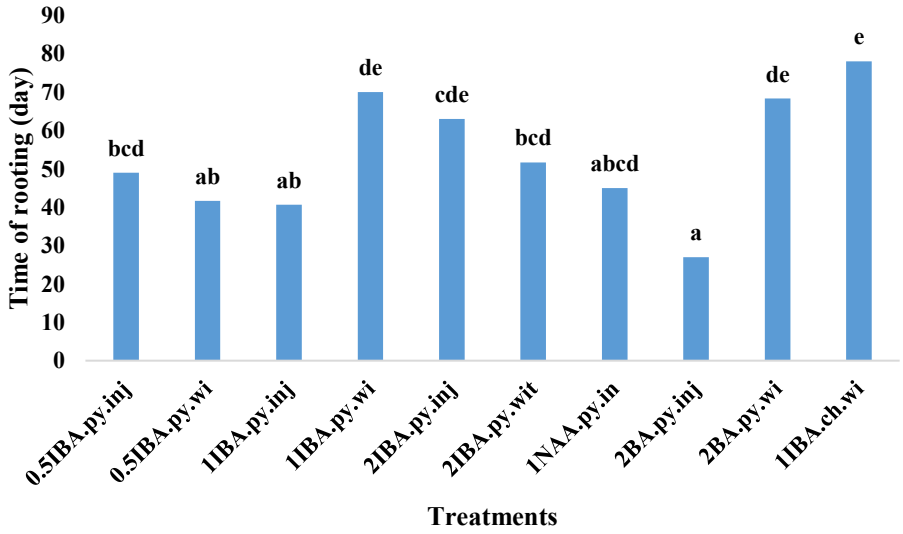


Figure 5. Rooting period in different treatments of japanese queen grafting on pyracantha under *in vitro* conditions. IBA: Indole-3-butyric acid, NAA: Naphthaleneacetic acid, BA: Benzyl adenine, py: *Pyracantha coccinea,* ch: *Chaenomeles japonica,* inj*:* medium injection wi*:* withouth medium injection

Evaluation of Rootstock and Culture Medium pH on the Growth of Micrografted Plants

The results shown highest scion growth (1.63 cm) was observed in C. *japonica* micrografts on C. *japonica* rootstock (Fig 6.). The greatest total plant length (2.53 cm) was recorded in micrografted explants cultured on medium adjusted

to pH 9.5 (Fig. 7). Furthermore, considering the interaction effect of pH and rootstock type, the greatest root length (4.33 cm) and number of roots (2.66) were observed in non-grafted C. *japonica* explants cultured on medium adjusted to pH 6.1. The highest chlorophyll b content (0.0040 mg g^{-1}) and carotenoid content (0.32mg g^{-1}) were recorded in non-grafted C. *japonica* explants cultured on medium with pH 5.9 (Table 2).

Table 2. The effect of culture medium pH and rootstock type on micrografted plants

Culture medium pH	Rootstock	Root length (cm)	Number of roots	Chlorophyll b (mg g^{-1})	Carotenoid (mg g^{-1})
5.7	*C. japonica* (non-grafted)	0.00 ^{c†}	0.00 ^{c}	0.0003 ^{d}	0.05 ^{e}
5.7	*C. japonica* micrografted onto *C. japonica* rootstock	3.40 ^{b}	1.33 ^{b}	0.0027 ^{bc}	0.19 ^{b}
5.7	*C. japonica* micrografted onto *P. coccinea* rootstock	0.00 ^{c}	0.00 ^{c}	0.0025 ^{bc}	0.16 ^{bc}
5.9	*C. japonica* (non-grafted)	0.00 ^{c}	0.00 ^{c}	0.0040 ^{a}	0.32 ^{a}
5.9	*C. japonica* micrografted onto *C. japonica* rootstock	0.00 ^{c}	0.00 ^{c}	0.0022 ^{c}	0.13 ^{cd}
5.9	*C. japonica* micrografted onto *P. coccinea* rootstock	0.33 ^{c}	0.66 ^{bc}	0.0021 ^{c}	0.12 ^{d}
6.1	*C. japonica* (non-grafted)	4.33 ^{a}	2.66 ^{a}	0.0043 ^{a}	0.16 ^{bc}
6.1	*C. japonica* micrografted onto *C. japonica* rootstock	0.00 ^{c}	0.00 ^{c}	0.0038 ^{a}	0.16 ^{bc}
6.1	*C. japonica* micrografted onto *P. coccinea* rootstock	0.00 ^{c}	0.00 ^{c}	0.0031 ^{b}	0.17 ^{bc}

†.Means within each column sharing at least one common letter are not significantly different according to Duncan's multiple range test.

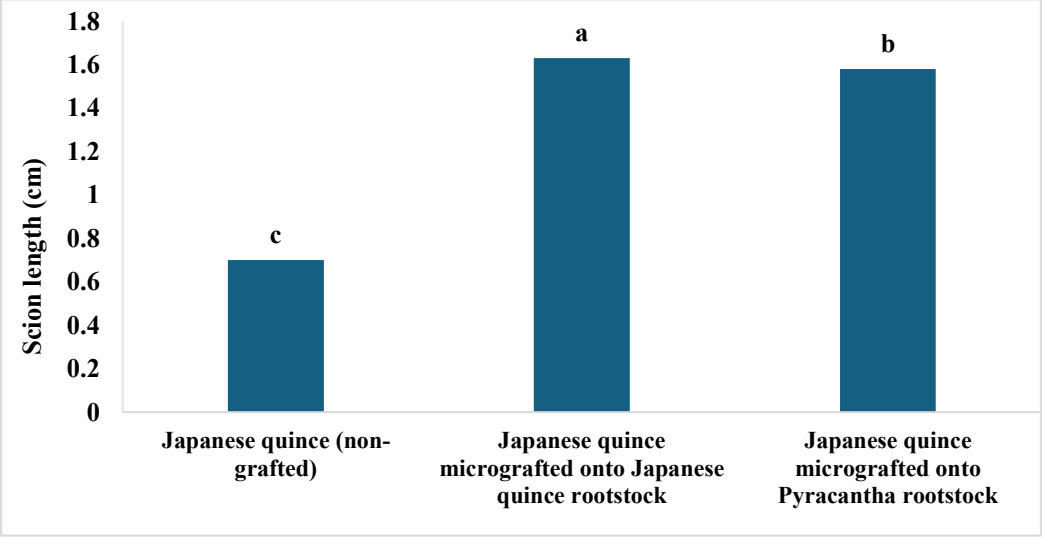


Figure 6. Effect of rootstock type on scion length under *in vitro* conditions

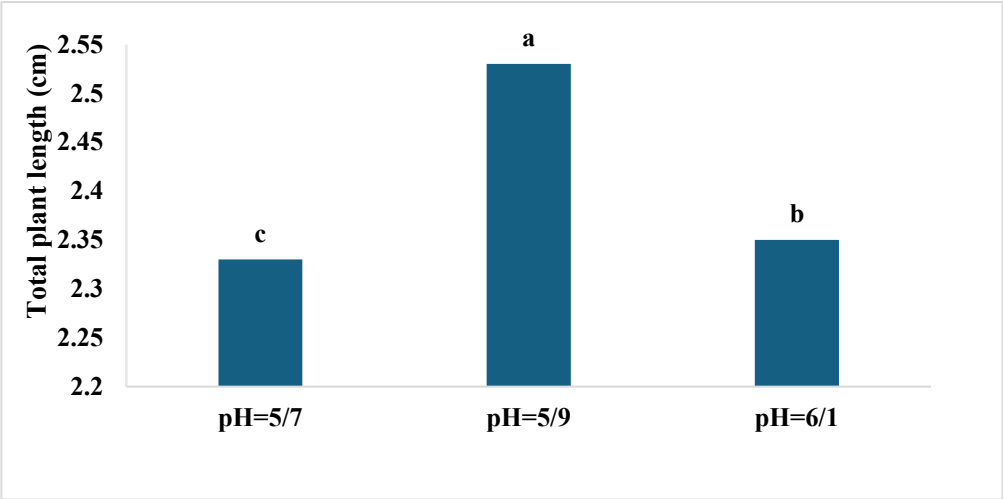


Figure 7. Effect of different culture medium pH levels on total plant length under *in vitro* conditions

The results of elemental analysis in micrografted and non-micrografted plants indicated that nutrient uptake varied among plants grown under different pH conditions. In terms of calcium, magnesium, sodium, and potassium uptake, micrografted plants on *P. coccinea* rootstock did not show superiority compared to non-grafted plants. However, a different pattern was observed for iron and manganese uptake; in all three pH levels of the culture medium, micrografted plants exhibited higher uptake of these elements than non-grafted plants. Specifically, iron uptake increased by 105%, 69%, and 306% in micrografted plants on *P. coccinea* rootstock compared to non-micrografted C. *japonica* at pH 7.5, 9.5, and 6.1, respectively (Fig. 8). Micrografted plants on *P. coccinea* rootstock showed increases in manganese uptake of 8%, 65%, and 1% at pH 5.7, 5.9, and 6.1, respectively, compared to non-micrografted plants (Fig. 9). Conversely, non-micrografted rootstocks were considerably more efficient in the uptake of calcium, sodium, magnesium, zinc and potassium than micrografted plants across the different culture medium pH levels (Fig. 10, 11, 12, 13 and 14).

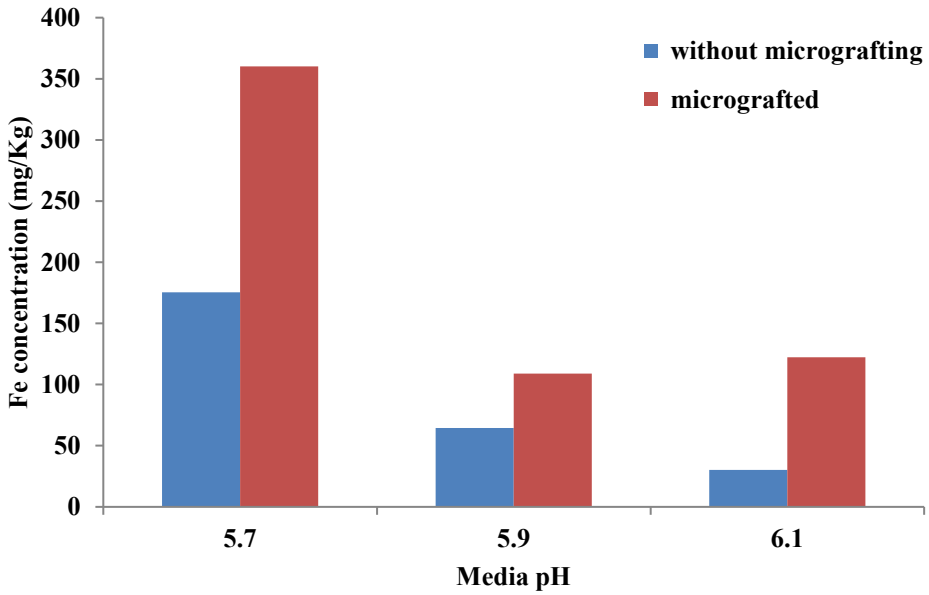


Figure 8. Effect of micrografting and culture medium pH on iron uptake in C. *japonica* under *in vitro* conditions

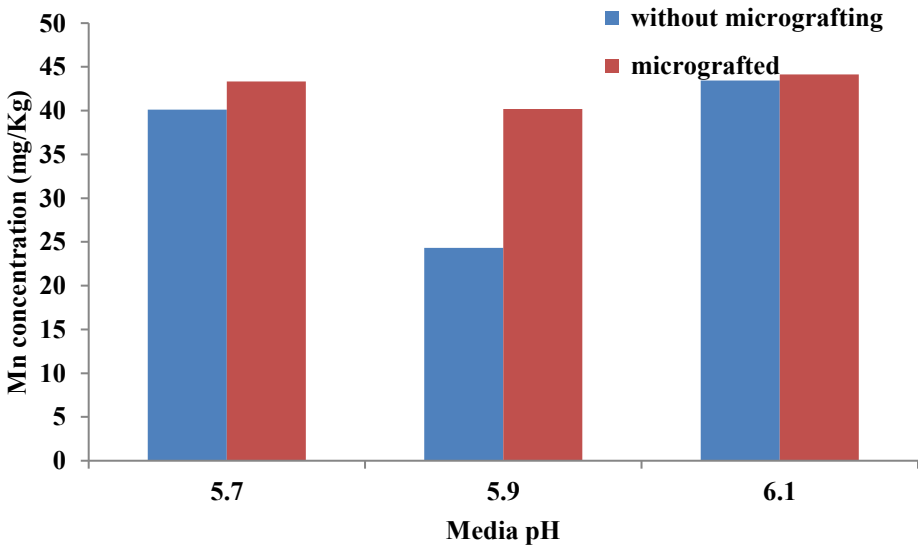


Figure 9. Effect of micrografting and culture medium pH on manganese uptake in C. *japonica* under *in vitro* conditions

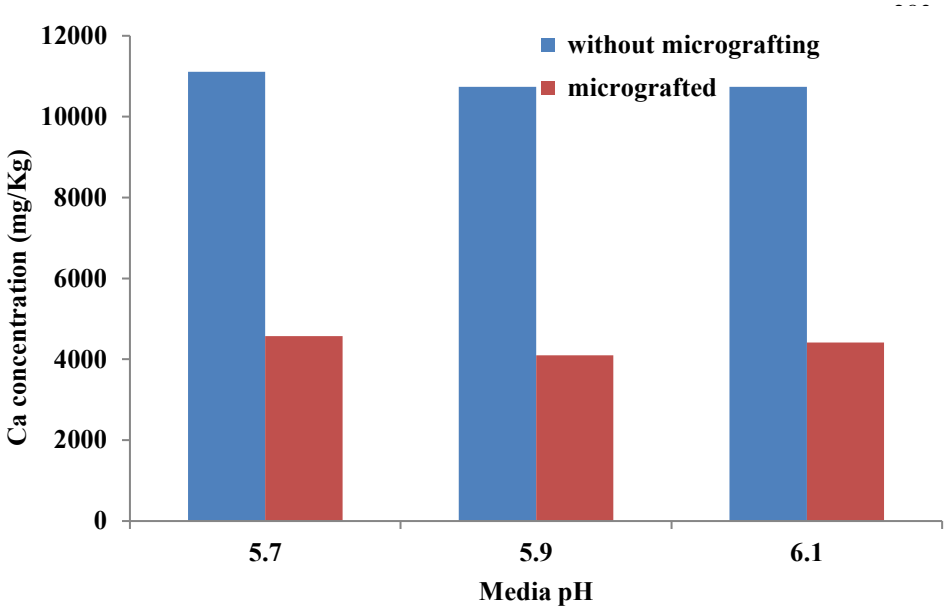


Figure 10. Effect of micrografting and culture medium pH on calcium uptake in c. *japonica* under *in vitro* conditions

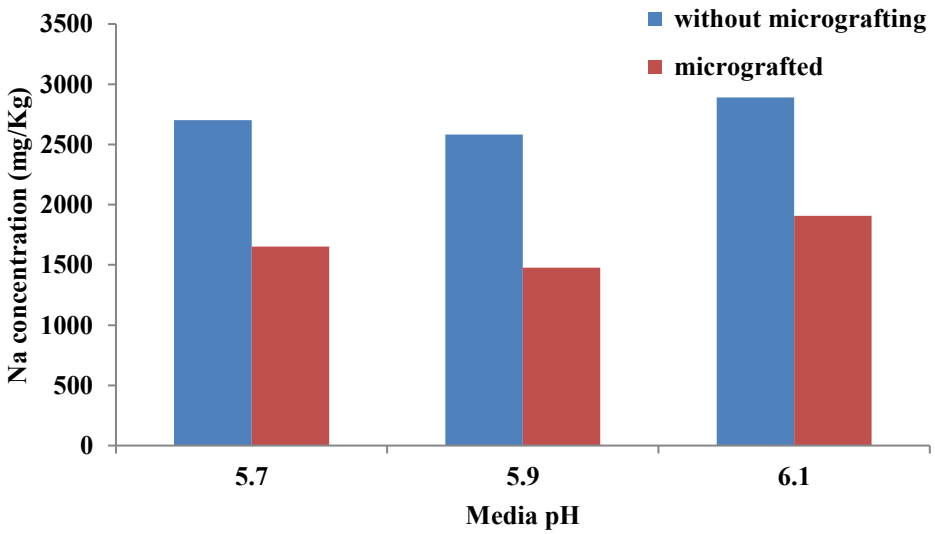
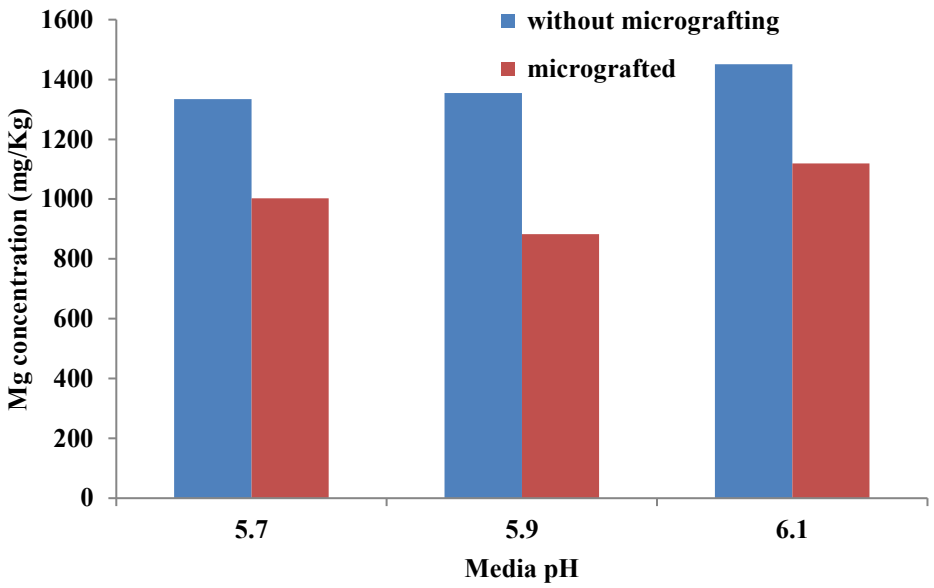


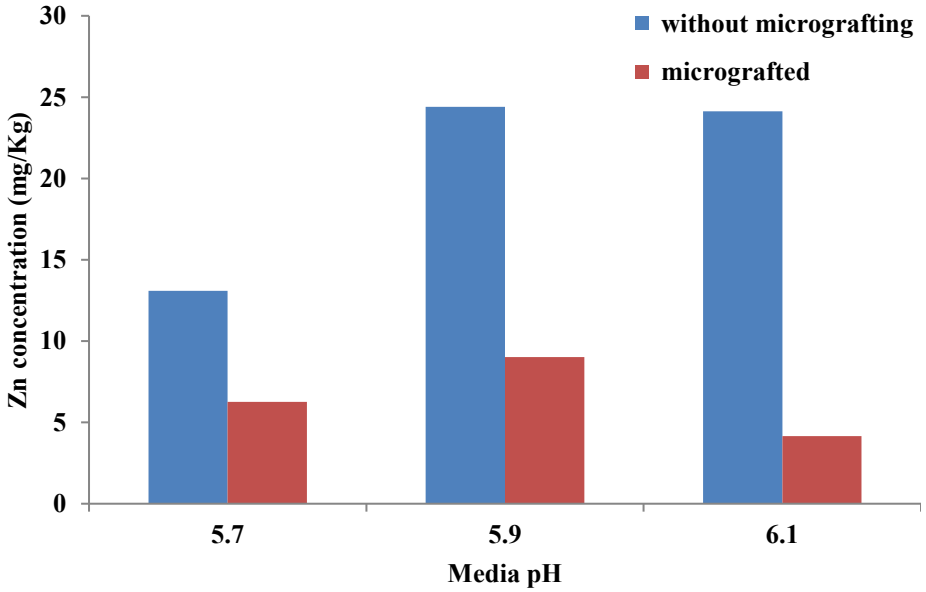
Figure 11. Effect of micrografting and culture medium pH on sodium uptake in C. *japonica* under *in vitro* conditions



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304 **Figure 12.** Effect of micrografting and culture medium pH on magnesium uptake in C. *japonica* under *in vitro*
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308 **Figure 13.** Effect of micrografting and culture medium pH on zinc uptake in C. *japonica* under *in vitro* conditions

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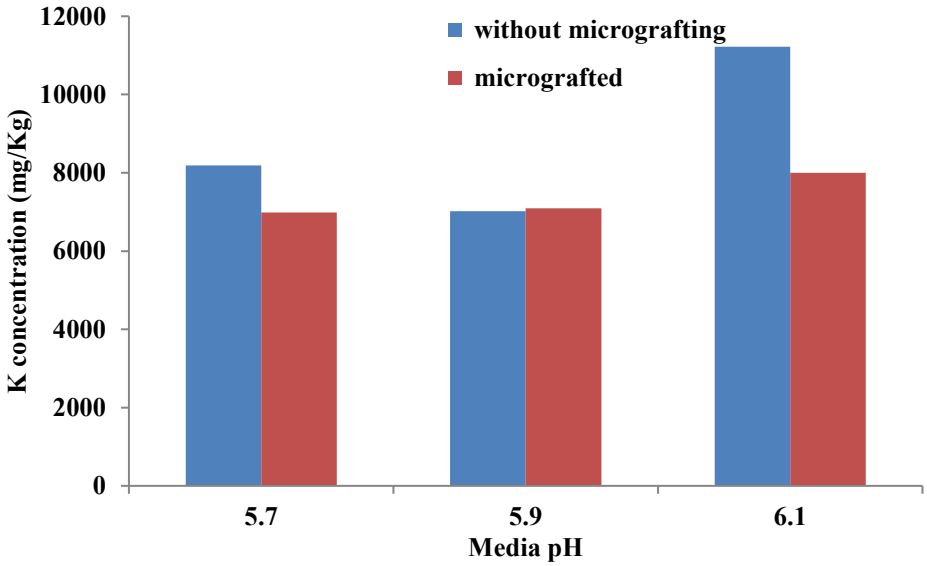


Figure 14. Effect of micrografting and culture medium pH on potassium uptake in C. *japonica* under *in vitro* conditions

Discussion

In the first experiment, the highest scion growth, scion greenness, rootstock greenness, callus formation at the graft union, and number of roots were simultaneously observed in C. *japonica* micrografts on *P. coccinea* rootstocks without culture medium injection at the graft union, cultured on half-strength MS medium supplemented with 0.5 mg l^{-1} IBA. Based on these results, rootstock type plays a decisive role in rooting and the successful establishment of micrografted plants due to interspecific genetic differences and variations in physiological compatibility. These factors can significantly influence scion establishment and growth by affecting the expression of genes related to hormonal pathways (especially auxin and cytokinin) and molecular mechanisms associated with stress responses. The success of C. *japonica* micrografting depends on various factors, including rootstock, the type of growth regulator used in the culture medium, and injection at the micrograft union. Studies such as those by Godeanu et al. (2004) and Taghizadeh et al. (2025) reported that vigorous and fast-growing rootstocks with high water and nutrient uptake capacity can enhance graft success through increased callus formation at the graft union. The findings of the present study also support this notion and demonstrate the effective role of *P. coccinea* rootstocks in improving graft establishment through enhanced callus formation and scion growth. Based on these results, *Pyracantha coccinea* rootstock can be considered suitable for C. *japonica* micrografting. Balanian et al. (2013) reported that in walnut, scion establishment and growth increase with enhanced uptake of soluble sugars by the rootstock. The vegetative trait findings of the present study are consistent with previous reports on pomegranate (Vazifeshenas et al. 2009), peach (Whiting et al., 2005), and cherry (Jimenez et al., 2006), confirming the role of the rootstock in regulating scion vegetative growth. In this study, the highest greenness of both rootstock and scion was observed in C. *japonica* micrografts on *P. coccinea* rootstock, which is likely due to reduced osmotic stress and improved hormonal balance, providing more favorable conditions for chlorophyll synthesis and overall physiological performance. Additionally, the synergistic effect of strong rooting in *P. coccinea* and the growth regulator IBA played an important role in improving greenness. This finding is consistent with previous reports on the combined effects of culture medium composition and rootstock on growth (Gonçalves et al. 2003; Peccoux et al. 2018). Weibel et al. (2003) also reported that the graft union, particularly the region near the connection site, can restrict or enhance scion vegetative growth by limiting the transport of water, growth regulators, and nutrients. Among the physiological factors affecting graft establishment, plant growth regulators are produced in both rootstock and scion tissues and are transported to the graft union. These compounds can play an important role in improving graft establishment and scion growth by affecting cell division and differentiation (Pinghai and Rongting 1993). According to the results of our experiment, the highest callus formation at the graft union occurred when no culture medium injection was applied. Under *in vitro* conditions, sufficient humidity is maintained around the graft union, and temperature and light are controllable. In addition, half-strength MS medium provides the rootstock with sufficient and controlled water and nutrients. Soheili-Vand et al. (2018)

reported that in *Citrus aurantifolia* micrografting, injection of MS nutrient solution containing agar into the graft union under *ex vitro* conditions in a growth chamber did not significantly affect graft establishment, callus formation, growth, or vigor of the seedlings compared to the control plants. This is likely due to the presence of sufficient humidity and controlled conditions in the culture chamber, which are similar to *in vitro* conditions and prevent moisture stress at the graft union. These results are consistent with previous findings. Moreover, the removal of MS culture medium from the graft union may act as a stimulatory factor for enhancing cell division and callus formation in the graft region. One possible explanation for this phenomenon is the induction of a mild physiological stress in plant tissues due to the absence of nutrient medium at the graft union. Weak stresses can activate signaling pathways related to cell division and re-differentiation (Thorpe 2007). In contrast, the presence of MS medium may provide a relatively stable and nutrient-rich environment, promoting the maintenance of normal cellular conditions and preventing the activation of stress responses and callus formation (George et al. 2008). Hormones play a fundamental role in regulating plant growth and differentiation. Auxin is one of the key phytohormones involved in rooting, particularly adventitious root formation. Auxin induces the formation of new roots by stimulating cell division and elongation in the differentiation zone. Additionally, this hormone activates the expression of genes associated with rooting signaling pathways. Exogenous application of auxins in plant tissue cultures increases rooting success (Pacurar et al. 2014). Izadi et al. (2013) considered rootability of the rootstock as an important factor influencing graft establishment and scion growth in the cutting-grafting method. Moreover, *P. coccinea* rootstock cultured on half-strength MS medium supplemented with 0.5 mg l^{-1} IBA produced a greater number of roots, resulting in enhanced growth and greenness of the micrografted plants. IBA, as one of the effective synthetic auxins, has been used historically and continues to be employed to improve rooting in cuttings of woody and semi-woody plants (Hartmann et al. 2011). The mechanism of IBA action involves increasing cell division in the cambial region and stimulating the differentiation of parenchyma cells into root cells, thereby initiating lateral root formation (Davies 2010). Similar studies have also shown that the application of IBA at low to moderate concentrations, particularly in the range of 0.5 to 2 mg l^{-1}, can enhance root formation in many ornamental plants, including barberry and *Rosa Canina* (De Klerk et al. 1999; Ahmad et al. 2014). Taghizadeh et al. (2024) also reported a positive effect of IBA on rooting, noting that the greatest root length and volume in Japanese quince grafted onto *Rosa Canina* rootstock were observed at a concentration of 2000 mg L^{-1} of this auxin. This indicates the effective role of this hormone in stimulating and enhancing root growth. In *in vitro* micropropagation of *P. coccinea*, IBA is generally the most effective and widely used auxin for rooting compared to other auxins (Nand et al., 2004). However, root formation can be influenced not only by the type of auxin but also by its concentration (Dong et al. 2017). Zare Khafri et al. (2021) reported that the highest number of roots in Iranian apricot cultivars was obtained in half-strength QL medium supplemented with 4 mg l^{-1} IBA. In *in vitro* peach culture, IBA at a concentration of 1.5 mg l^{-1} induced the highest rooting rate (42.86%), the greatest number of roots (6.33 roots per cutting), and the longest roots (17.7 cm) (Felek et al. 2017). Sahari et al. (2022) reported that the highest number and length of roots in *P. coccinea* under *in vitro* conditions were obtained on half-strength MS medium containing 1 and 1.5 mg l^{-1} IBA. These results are consistent with our findings regarding the use of IBA for rooting *P. coccinea* explants; however, considering the rootstock type and the *in vitro* grafting process, a lower concentration of IBA was required for rooting compared to the concentrations used in other studies.

In classical plant physiology, cytokinins such as BAP generally suppress root induction because they promote shoot development (Hartmann et al. 2011). However, in micrografting systems, local cytokinin activity can accelerate callus and cambial bridge formation, establishing early vascular reconnection between scion and rootstock. This rapid callogenic response supports earlier root primordia formation once auxin flux resumes at the graft interface (George and De Klerk 2008). BAP‐induced accumulation of polyamines (e.g., putrescine and spermidine) may also enhance tissue repair and mitigate oxidative stress, thereby indirectly facilitating root initiation (Ahkami et al. 2009). Auxin treatments (IBA and NAA) exhibited the expected dominance for root induction, but their efficiency depended critically on both concentration and delivery method. Direct injection of IBA produced faster rooting than equivalent non‐injected treatments, suggesting that hormone diffusion through the solid culture medium was rate‐limiting. Local accumulation of IBA at the graft site likely triggered auxin‐response gene expression and cell reprogramming toward root meristem identity (De Klerk et al. 1999; Ludwig‐Müller 2011). In contrast, high IBA concentrations (2 mg L^{-1}) slowed rooting, probably due to excessive ethylene and phenolic compound synthesis that interferes with initial root organogenesis, consistent with the inhibitory threshold reported by Gaspar et al. (2003). Naphthaleneacetic acid (NAA) at 1 mg·L^{-1} yielded intermediate rooting speed. NAA is a more active but less stable auxin analogue, producing a rapid yet transient effect on root induction. Although effective when injected, its shorter persistence compared with IBA may account for the slower overall rooting relative to the optimal IBA treatment. Across all treatments, medium injection into the graft union consistently improved rooting performance. This procedure evidently enhanced nutrient

and hormone availability at the cambial interface, supported rapid wound healing, and prevented local desiccation or necrosis — phenomena previously reported in liquid or semi‑liquid culture systems (Alvard et al. 1993).

The rootstock plays a fundamental role in the growth and performance of the scion in micrografted plants. Differences in physiological characteristics of rootstocks can affect water and nutrient uptake as well as the plant's tolerance to environmental stresses. According to Warschefsky et al. (2016), selecting a suitable rootstock for grafting improves scion growth and enhances overall plant performance. Moreover, compatibility between rootstock and scion is essential for the effective transport of hormones and nutrients. Changes in the pH of the culture medium can significantly influence the growth and development of different plant rootstocks, which in turn affects scion acceptance and growth. An appropriate culture medium pH increases nutrient uptake and improves enzyme activity in the rootstock, thereby promoting better physiological compatibility with the scion (Singha et al. 1987). Moreover, rootstock responses to pH can be species or cultivar-dependent, which further emphasizes the importance of selecting a suitable rootstock (Miller and Murashige 1976). According to the results of the present experiment, the greatest scion growth was observed on C. *japonica* rootstock. The pH of the culture medium plays a crucial role in plant tissue culture. Different plants require different pH levels, and excessively high or low pH can negatively affect normal plant growth and development. Furthermore, when pH is improperly adjusted, the culture medium may become either too hard or too soft, which is not conducive to the normal growth of tissue culture explants, as it affects nutrient uptake through the roots and subsequently impairs plant growth (Taj et al. 2019). Pasqua et al. (2002) reported that tobacco explants cultured under low pH conditions (4–5) produced a greater number of longer shoots. In the second experiment, increasing the culture medium pH resulted in reduced shoot height and number. Sharma et al. (2018) reported that changes in medium pH in *in vitro Saccharum officinarum* culture significantly affect shoot formation, and that shoot proliferation rate and growth are influenced by medium pH. The differences observed among various studies can be attributed to genetic variations between species or plant genotypes. As noted by George and Sherrington (1984), plant physiological responses to environmental conditions, including pH, are strongly genotype‑dependent, and each species or genotype has an optimal pH range for its *in vitro* growth. Therefore, it can be concluded that determining the optimal pH for *in vitro* culture media should be performed specifically for each plant species or genotype to provide suitable conditions for vegetative growth. Various studies have shown that culture medium pH is one of the key factors in inducing rooting in *in vitro* culture. De Klerk et al. (2008) reported that in various plants, including *Prunus avium* L, culture media with pH below 5 increased rooting percentage, whereas increasing the medium pH significantly reduced rooting. This effect is likely related to the regulation of hydrogen ions at the cellular level and their influence on the uptake of hormones such as auxin. However, the results of Yao et al. (2022) on *Actinidia chinensis* explants showed that the highest graft establishment and rooting percentage were achieved at pH 6 in half-strength MS medium, which is in agreement with the findings of the present study. In second experiment, the highest root number and root length were observed in non-grafted explants at pH 6.1, indicating an optimal response of this genotype under near-neutral conditions. The differences between these reports can be explained by considering genotypic variations and the hormonal regulation mechanisms of each species. As Gaspar et al. (1996) stated, plant responses to culture medium pH strongly depend on species, explant type (leaf, stem, node), and even the developmental stage of the plant. Moreover, pH can affect the availability of mineral nutrients and the uptake of hormones, particularly auxins, which play a crucial role in adventitious rooting (Salisbury and Ross 1992). Based on these points, it can be concluded that there is no single optimal pH suitable for all plant species, and each genotype requires a specific pH for optimal rooting induction depending on its physiological characteristics. In the present study, the highest chlorophyll b and carotenoid contents were observed in non-grafted C. *japonica* plants cultured on medium with a pH of 9.5. This finding indicates a significant effect of culture medium pH on the synthesis of photosynthetic pigments. Nutrient uptake from the culture medium also differs among rootstocks, leading to variations in chlorophyll and carotenoid levels (Mosa et al. 2022). Although iron is not directly present in the molecular structure of chlorophyll, its essential role in enzymatic processes related to chlorophyll synthesis means that adequate iron supply enhances chlorophyll production. Consequently, chlorophyll status can have a substantial impact on photosynthetic activity. Previous studies have also highlighted the importance of pH in regulating pigment biosynthesis. For example, Zhao et al. (2013) reported in *Arabidopsis thaliana* that low pH values within an optimal range (5.5–6.0) increase chlorophyll and carotenoid content, while deviation from this range reduces pigment synthesis and lowers photosynthetic capacity. Furthermore, Taiz and Zeiger (2015) indicated that root-zone pH regulation can significantly influence chlorophyll production by affecting the uptake of mineral nutrients such as magnesium, which is the central element of the chlorophyll molecule. The increase in carotenoid content may also be related to the protective role of these pigments under optimal pH conditions. In addition to their role in photosynthesis, carotenoids protect chlorophyll from oxidative damage (Demmig-Adams and Adams 1996). Therefore, the provision of suitable conditions for pigment synthesis may indicate a healthy photosynthetic status of the cells in the growth medium at pH 9.5. Overall, the findings of this study are in line with previous research and emphasize that precise adjustment of culture medium

pH is a key factor in improving the physiological quality and pigment content of plants. The significant interaction between PGR concentration and substrate pH underscores the complexity of vascular reconnection. Cytokinins (e.g., BAP) are known to accelerate vascular differentiation; however, our data indicate that their efficacy is strictly pH-dependent. At sub-optimal pH levels, the metabolic cost of maintaining vascular integrity might overshadow the growth-promoting effects of these regulators, explaining the reduced survival rates observed in our control groups. This indicates a 'physiological trade-off' where excessive hormonal stimulation cannot compensate for nutrient-deficiency stress.

In second experiment, different rootstocks exhibit selective nutrient uptake, meaning that some rootstocks have a greater affinity for certain elements depending on various factors. The results indicated an enhanced iron uptake efficiency in C. *japonica* when micrografted onto *P. coccinea* rootstock, particularly under alkaline culture medium conditions. Taghizadeh et al. (2025) reported that C. *japonica* grafted onto *P. coccinea* rootstock showed higher iron uptake under alkaline soil conditions compared to C. *japonica* grafted onto its own rootstock, which is consistent with the findings of the present study. In the present experiment, *P. coccinea* rootstock absorbed more iron than *C. japonica* rootstock, suggesting that the root system of *P. coccinea* is capable of activating mechanisms that create favorable conditions for enhanced iron uptake from the growing medium. These mechanisms may include modification of the rhizosphere pH, alteration of apoplastic pH in roots, and increased activity of root ferric chelate reductase (FCR). One of the plant responses to iron deficiency is the extrusion of H^+ ions via H^+-ATPase, which leads to rhizosphere acidification. Rhizosphere pH has significant effects on nutrient mobility and microbial activity. A reduction in rhizosphere pH increases the activity of root ferric chelate reductase, resulting in the reduction of Fe^{3+} to Fe^{2+}. Depending on the plant's nutritional status, soil buffering capacity, genotype, and rhizosphere pH, the rhizosphere pH may differ by up to two units from the bulk soil pH. Acidification of the soil–root interface is a response induced by iron deficiency and is mediated by the proton pumps of the AHA family (Arabidopsis H^+-ATPase), which lower the medium pH and increase the solubility of inorganic iron. Numerous studies have confirmed that in iron-deficient roots, both FCR activity and proton extrusion are higher compared to roots exposed to sufficient iron. Rhizosphere acidification by plasma membrane–associated H^+-ATPase increases Fe^{3+} solubility, and Fe^{3+} is reduced to Fe^{2+} by FCR. The uptake of Fe^{2+} is then regulated by the IRT1 transporter located in the root epidermis (Krohling et al. 2016). The enhancement of iron uptake at lower pH is likely linked to the induction of the Strategy I iron-acquisition mechanism. In *Chaenomeles* species, which are prone to chlorosis, maintaining an acidic environment in the micro-environment of the graft union may favor the activity of plasma membrane-bound ferric-chelate reductase (FRO2). We hypothesize that the application of IBA or BAP further modulates the expression of genes associated with iron transport *IRT1*, thereby preventing the systemic chlorosis typically observed in neutral or alkaline media. This suggests that the graft union acts not merely as a physical connection, but as a physiological gatekeeper for ion transport. Our findings demonstrate that micrografting efficiency and subsequent physiological health of *Chaenomeles japonica* are significantly regulated by the synergism between the nutrient-limiting pH and exogenous hormonal balance. The observed increase in survival rate and reduction in iron-deficiency chlorosis at specific lower pH levels suggests a dynamic regulation of nutrient bioavailability, potentially mediating the uptake pathways at the graft union. These results corroborate and extend previous findings by (Zhao et al. 2023) regarding the critical role of the rhizosphere/apoplast acidification in rootstock performance.

Conclusion

The success of grafting in in vitro micrografting of C. *japonica* onto *P. coccinea* rootstock is significantly affected by both the hormonal balance and the method of hormone delivery employed. The physical process of injecting the culture medium did not consistently ensure superior outcomes. In numerous cases, treatments that did not involve injection yielded the highest scion growth scores (for instance, P. *coccinea* with 0.5 mg l^{-1} IBA without injection). This indicates that excessive contact with the medium or the stress caused by injection may be harmful under specific plant growth regulator (PGR) conditions. The most effective scenario was observed with the injected medium containing 2 mg l^{-1} BAP or 1 mg l^{-1} IBA, which resulted in the quickest root emergence. An optimal concentration of auxin (approximately 1 mg l^{-1} IBA) seems to be crucial, as higher concentrations can delay root development. The injection of culture medium significantly enhances the uptake of hormones and accelerates the formation of graft unions and root initiation. These results underscore the functional synergy between localized cytokinin-driven callus formation and auxin-regulated root organogenesis in micrografting settings. Such findings can inform the optimization of hormonal formulations and delivery methods to improve success rates in the clonal propagation of woody species. The condition of pH 5.7 was found to be the most effective for initiating roots in the compatible graft combination. In contrast, the pH 5.9 condition led to the highest levels of Chlorophyll b and Carotenoid in the non-grafted control

plants, indicating that this pH is optimal for sustaining overall plant health and pigmentation in undisturbed tissue. However, this pH did not result in successful rooting when applied to the grafted material. Micrografting appears to place considerable stress on the newly developed plant, which may interfere with the effective transport and absorption mechanisms at the outset, resulting in a diminished accumulation of essential elements (such as Calcium and Potassium) in micrografted tissues when compared to non-grafted controls. The effectiveness of nutrient mobilization is closely linked to the health and functionality of the graft union. Therefore, protocols for establishing micrografts should include nutrient formulations that specifically address any deficiencies observed as a result of the grafting process, which may necessitate higher concentrations of limiting ions like Ca. Micrografted plants utilizing *P. coccinea* coccinea rootstock consistently demonstrated enhanced uptake of Iron and Manganese relative to non-grafted controls across all pH levels tested. For example, Iron uptake was observed to increase significantly at pH 6.1 in micrografted plants, suggesting that the *P. coccinea* rootstock is markedly more effective at mobilizing these particular micronutrients when used in conjunction with the micrografting technique. In conclusion, achieving successful micrografting of C. *japonica* under in vitro conditions necessitates a careful equilibrium: the pH of the culture medium must be optimized not only for general nutrient solubility but also to specifically support the impaired ion transport system of the developing graft union. The experimental findings strongly indicate that employing the *P. coccinea* rootstock for micrografting C. *japonica* could serve as an effective approach to alleviate iron chlorosis, a prevalent issue in alkaline soils that diminishes chlorophyll content. Notably, the observation that the micrografting significantly improves the uptake of Iron and Manganese suggests a functional modification in the nutrient acquisition pathways that are transferred from the root system to the scion. This targeted nutrient enhancement is crucial, as iron chlorosis arises directly from the plant's inability to absorb adequate iron under elevated pH conditions. Therefore, this micrografting approach offers a physiological mechanism to bypass environmental iron immobilization, potentially leading to healthier, greener foliage by ensuring adequate Fe supply to the scion tissue. Future research can leverage this by developing a compensatory two-stage nutrient regime—one to exploit the Fe/Mn enhancement and another to correct the reduced uptake of other key elements—thereby optimizing overall plant health. In conclusion, our results provide a refined protocol for the successful micrografting of *C. japonica*. By fine-tuning the chemical environment at the graft union, we can mitigate iron-deficiency symptoms at the nursery stage. Future studies utilizing RT-qPCR to analyze the expression levels of *FRO2* and *IRT1* under these specific conditions will be essential to elucidate the exact molecular mechanisms underlying the observed phenotypes.

Statements and Declarations

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Author contribution MT conceived and designed the research approach. NSH assembled the experimental setup and conducted the experiments. MT analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval

Not applicable, as this study did not involve human or animal subjects.

Competing interests The authors declare that they have no competing interests.

Funding Declaration

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