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Dual Optimization for Enhanced Micrografting Efficiency of Chaenomeles japonica L.: Rootstock Selection, Medium Injection, and Leaf Fall Mitigation Strategies

Mina Taghizadeh

m-taghizadeh@araku.ac.ir

Arak University

Negin Sadat Hosseini

Arak University

Research Article

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Abstract

Background

Chaenomeles japonica L. serves as a multi-functional landscape shrub respected for its visual attraction and durability. Alkaline soils can lead to leaf chlorosis or yellowing of young leaves during the growing season. The cost-effective and efficient approach is to employ varieties that exhibit resistance to iron chlorosis. This study aimed to enhance the *in vitro* micrografting process of *Chaenomeles japonica* and composition of the culture medium to mitigate leaf abscission. In the first experiment investigated the effect of grafting time, rootstock type, and the injection of culture medium at the graft union. The second experimental treatments comprised: vitamin C, silver nitrate, iron 2X and 4X concentration.

Results

The findings indicated that the highest of callus formation was observed in late October. The rootstock type proved a critical determinant of micrograft success. Rootstock type significantly affected both scion growth and callus formation, highlighting its pivotal role in graft compatibility. Also, Silver nitrate (10 mg L^{-1}) is the most effective treatment for eliminating all post-subculture physiological disorders in micrografted *Chaenomeles japonica*. Increasing Fe concentration reduces chlorosis but does not eliminate wilting or leaf fall and Vitamin C alone is not significantly effective.

Conclusions

Rootstock type significantly affected both scion growth and callus formation, highlighting its pivotal role in graft compatibility. The injection of culture medium, although not influential on growth or greenness attributes, exerted a significant impact on callus formation at the graft interface, indicating a potential physiological or biochemical modulation of wound-healing processes. Silver nitrate is the most effective treatment for eliminating all post-subculture physiological disorders in micrografted *Chaenomeles japonica*.

Background

Chaenomeles japonica L., commonly known as Japanese quince, is a deciduous shrub indigenous to Japan, extensively utilized in ornamental horticulture and landscape due to its propensity for early flowering, vivid coloration, adaptability, and ecological advantages. As a member of the Rosaceae family, this species merges significant aesthetic appeal with functional landscape roles, rendering it a versatile choice for both urban and natural environments. The Japanese quince is recognized as one of the first flowering shrubs, producing thick clusters of flowers in hues of red, pink, orange, or white before the leaves begin to appear. This characteristic positions it as a crucial species for infusing color into late winter and early spring landscapes [1]. *Chaenomeles japonica* serves as a multi-functional landscape

shrub respected for its visual attraction, durability, and ecological contributions. Its capacity to thrive under urban stress, early flowering display, and benefits to wildlife establish it as a vital element in sustainable and biodiverse landscape designs. This ornamental shrub flourishes optimally in soils that are neutral to slightly acidic. In contrast, alkaline or calcareous soils can lead to leaf chlorosis or yellowing of young leaves during the growing season, attributed to the impaired iron absorption by *Chaenomeles japonica* [2, 3]. Symptoms of iron deficiency in plants grown in calcareous soil manifest as yellowing of leaves, alterations in the plant's iron concentration, and a reduction in the dry weight of both roots and shoots [4]. To address this issue, several strategies have been proposed, including modifying soil pH, applying iron-containing fertilizers through soil application or foliar spraying, injecting them into tree trunks, utilizing iron citrate compounds, or lowering soil pH by incorporating sulfuric acid [5, 6]. Each of these methods presents a range of advantages and disadvantages [7]. The most cost-effective and efficient approach is to employ varieties that exhibit resistance to iron chlorosis [8, 3]. Iron absorption in plants is governed by genetic factors, which means that when various cultivars and varieties of the same species are cultivated under identical conditions, their responses to iron absorption and nutritional needs will differ [9].

Plants are primarily propagated through two techniques: sexual and asexual. Sexual propagation presents challenges such as increased heterozygosity, prolonged seedling duration, seed-borne pathogens, loss of rootstock benefits, and inferior yield quality. Consequently, vegetative propagation techniques like grafting, cuttings, and layering are predominantly employed in horticultural crops [10]. *In vitro* micrografting serves as a significant technique for the micropropagation of a diverse range of plant species, particularly those that are woody. Over recent decades, *in vitro* micrografting has emerged as a method to enhance shoot regeneration and the adaptation of horticultural species cultivated *in vitro* [11]. This process is conducted in a humid, sterile, and precisely controlled environment. The micrografts and rootstocks are free from pathogens in *in vitro* cultures. Successful grafting relies on the formation of callus and rapid vascular connections between the scion and rootstock [12, 13]. Micrografting integrates plant tissue culture with grafting method, allowing for the combination of tissue-cultured rootstock with scion in *in vitro* settings [14]. In contrast to conventional grafting, *in vitro* micrografting is characterized by the smaller sizes of scion and rootstock, as well as the maintenance of sterile conditions throughout the grafting procedure. This method offers numerous benefits, including continuous plant production, elimination of viruses, compatibility of grafts [13]. Given that the evaluation of graft incompatibility in grafted trees can extent months to years, micrografting proves to be a valuable approach for anticipating graft incompatibility during the initial growth phases of horticultural crops [15]. The effectiveness of the micrografting technique is contingent upon the successful integration of the rootstock and scion tissues. Ability in grafting is a critical element influencing the success of *in vitro* grafting [16, 17]. The efficiency of micrografting is influenced by the culture medium, the species of the plant, and the origin of the plant material utilized. Additionally, growth regulators present in the culture medium can impact the success of micrografting. The plant growth regulations most frequently employed in micrografting cultures are auxins and cytokinins [18]. The composition of the culture media is another crucial factor determining the compatibility of micrografting. The balance between cytokinin and auxin concentration,

the source of carbohydrates, and the addition of anti-ethylene agents such as silver nitrate ($AgNO_3$) can strongly influence the physiological stability of the scion. The accumulation of ethylene within sealed culture vessels frequently results in leaf chlorosis and abscission, which can lead to reduced survival rates of the grafted plantlets [19]. $AgNO_3$, functioning as an ethylene action inhibitor, has been effectively utilized in various woody species to avoid leaf senescence and enhance chlorophyll preservation during micropropagation [20]. The Role of vitamin C in plant *in vitro* culture or ascorbic acid, is vital for numerous physiological functions in plants. Within *in vitro* culture systems, vitamin C acts not only as a growth enhancer but also as an antioxidant and an income of stress resistance. The incorporation of vitamin C in plant tissue culture has attracted considerable interest due to its diverse beneficial impacts on plant growth, development, and regeneration [21]. The browning of plant tissues, especially callus and explants, is a prevalent challenge in tissue culture. This phenomenon is attributed to the oxidation of phenolic compounds, which can result in tissue necrosis and impede growth. As an antioxidant, vitamin C plays a crucial role in mitigating these adverse effects. As an antioxidant, vitamin C plays a crucial role in preventing the oxidative browning of explants by inhibiting the enzymes responsible for phenolic oxidation, including polyphenol oxidase [22]. By preserving the color and vigor of plant tissues, vitamin C contributes to a more successful and extended culture period, thereby enhancing the overall health and vigor of the plant material [23].

In addition to the success of graft unions and leaf retention, the translocation of nutrients across the graft interface is essential for the overall performance of regenerated plants. Recent studies indicate that micrografting can serve as a biotechnological approach to mitigate micronutrient deficiencies, particularly iron deficiency, by employing rootstocks with improved uptake capabilities [24]. Iron deficiency is a prevalent issue in tissue culture systems due to the limited availability of bioavailable Fe^{3+} and instabilities in pH within the medium. The incorporation of iron-efficient rootstocks in *Chaenomeles japonica* micrografting may enhance chlorophyll biosynthesis and plant vigor, presenting a novel strategy for maintaining physiological balance under *in vitro* conditions. Micrografting has emerged as a critical technique in plant tissue culture, providing a precise and controlled method for the combination of scion and rootstock tissues in aseptic *in vitro* environments. This technique has garnered increasing interest for woody ornamental species such as *Chaenomeles japonica* (Japanese quince), which is valued for both its ornamental and medicinal properties but encounters propagation challenges due to physiological dormancy and rooting limitations [25, 26]. The species' potential as a source of bioactive compounds and its tolerance to abiotic stress reduce it a promising candidate for biotechnological propagation and genetic enhancement [1]. Ongoing breeding efforts and its application in ecological landscaping will further its role in climate-resilient urban landscapes.

Nevertheless, despite these advancements, the optimization for *C. japonica* remains significantly under-researched. This research aimed to enhance the *in vitro* micrografting process of *Chaenomeles japonica* by assessing the synergistic effects of seasonal timing, rootstock variety, and media injection at the grafting union. Furthermore, it explores the composition of the culture medium, which includes the use of silver nitrate, vitamin C, and elevated levels of Fe to mitigate ethylene-induced leaf abscission. By examining these interrelated physiological and biochemical elements, the study offers novel insights into

the formulation of sustainable and reproducible propagation protocols for woody ornamentals in *in vitro* culture.

Materials

Plant materials

In this study, the plant species Pyracantha (*Pyracantha coccinea*) was utilized as a rootstock, while Japanese quince (*Chaenomeles japonica*) served as the scion. The plant materials for this research were sourced from cultivated plants in Arak municipal landscapes, with all cuttings derived from a single plant stock and propagated through semi-woody cuttings in the research greenhouse of the Horticultural Department at Arak University, Iran, under humidity conditions of 50-60% and temperatures of 25°C–30°C, 16L:8D photoperiod and 10,000 lux of light. The cuttings were prepared in early spring, measuring 15-20 cm in length, and were rooted in a moist perlite substrate. After a period of 3 months, the rooted plants were transferred to pots containing a substrate composed of a mixture of agricultural soil and manure (1:1).

Disinfection 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 pyracantha branches (serving as rootstocks) measured 3-4 cm in length and possessed a minimum of two vegetative buds, while the Japanese quince branches (acting as scions) were 1 cm long and contained one vegetative bud. The plant material underwent a thorough wash under running water with 2-3 falls of Tween 20 (2–3 falls L^{-1}) for 30 min, followed by immersion in tobacco extract for 30 min [27]. The explants were submerged in solution of 20% (v/v) home commercial bleach (Golrang company) 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.

Experiment 1- Effect of time, rootstock type, and culture medium injection on micrograft success

Preparation of culture medium

The level of endogenous bacterial contamination and phenol secretion in Pyracantha tissues was significantly elevated according to a pretreatments experiment conducted during the disinfection phase (data not presented). Consequently, the antibiotic of meropenem (control of *in vitro* pathogen) and activated charcoal (AC) (to inhibit phenol secretion from explants) were incorporated into the culture medium to mitigate this adverse sign. The culture medium utilized in this investigation consisted of half-concentration MS supplemented with 0.5 mg L^{-1} IBA, 15 mg L^{-1} sucrose, 4 mg L^{-1} AC, and 7 mg L^{-1} agar. The pH of the culture medium was adjusted to a range of 7.5 to 8.5 using NaOH 0.1 N. The culture

medium autoclaved at 121 ± 1°C and 1.2 atmosphere pressures for a duration of 20 minutes. After the culture medium was sterilized, once its temperature cooled to approximately 45°C, 100 mg L^{-1} of the antibiotic meropenem was introduced into the culture medium and thoroughly mixed.

Experimental Methods and treatments

Based on the pretreatments performed, it was found that the cleft grafting method was employed due to minimizes displacement and misalignment between the rootstock and scion (data not shown). Thereby reducing the probability of fracture at the grafting union and browning of the graft. Furthermore, to ensure the stability of the tissues at the grafting union and to preserve moisture necessary for callus formation and the establishment of a connection between the rootstock and scion, it has been recommended to apply a coating at the grafting union [28]. Consequently, the culture medium utilized for plant establishment was injected directly into the grafting union. So, this experiment investigated the effect of grafting time (mid-October, late October, and mid-November), rootstock type (Japanese quince micrograft on Japanese quince rootstock and Japanese quince micrograft on Pyracantha rootstock), and the injection of culture medium at the graft union (injection and non-injection) on the success of micrografts. The experimental was conducted a completely randomized design as factorial arrangement with three replications for each treatment. Micrografting for the grafting process, 3-4 mm long shoot segments of Pyracantha that have two vegetative buds and 2-3 leaves (to provide nutrition for the rootstock) were selected as rootstocks, while 1 cm Japanese quince shoots with a single vegetative bud were selected as scions. Initially, a rootstock and scion of matching diameter (approximately 2 mm) were chosen. The grafting method employed in this study was a cleft graft. A vertical slit measuring 3 to 5 mm was made on the rootstock using a scalpel. A V-shaped cut was then created at the end of the prepared scion, which was subsequently inserted into the gap formed in the rootstock, ensuring that the vascular cambium of both the rootstock and scion were perfectly aligned. Following the connection of the rootstock and scion, approximately 0.1 ml of sterilized culture medium at a temperature of 45 °C (prior to the solidification of the culture medium) was injected into the graft union using a sterile syringe. Ultimately, the grafted plants were kept in a growth chamber maintained at a temperature of 19 ± 1 °C, with a light intensity of 60 µmol m^2 s^{-1}, and a photoperiod consisting of 16 hours of light and 8 hours of darkness. Throughout the growth period, the sterilized culture medium was re-injected into the graft union three times, at two-week intervals (Fig. 1).

Characteristics measurement

Measurement of traits beginning in the first week of cultivation. Visual traits such as callus formation and the success of micrografting were assessed, while other traits of the micrografting plants were evaluated eight weeks after micrografting operation. Callus scoring was conducted using a coding system. To evaluate the callus rate, codes 0, 1, 2, and 3 were assigned for the absence of callus, minimal callus, average callus, and maximum callus formation, respectively. Traits associated with visual growth rate of rootstock and scion were recorded using scores of 1, 2, 3, 4, and 5. Here, code 1 indicated the lowest growth rate of length, while code 5 represented the highest growth length. Traits related to the

greenness of the rootstock and scion were evaluated based on visual characteristics, including color, freshness, and the appearance of leaves from both the rootstock and scion, using scores of 1, 2, 3, 4, and 5. In this scoring system, code 1 denoted the lowest level of greenness, whereas code 5 indicated the highest level of greenness.

Experiment 2- Eliminating the effects of leaf senescence during the subculture of micrografted plants

The leaves initially exhibited yellowing, subsequently withering and falling off during the subculture phases of micrografted plants (Fig. 1-e). This issue ultimately led to the death all of the micrografted plants and preventing further progression in the propagation stages. So, a variety of compounds incorporated into the culture medium were evaluated to address this adverse effect in this experiment. The experimental treatments comprised: 1000 mg L^{-1} of vitamin C, 10 mg L^{-1} of silver nitrate, iron 2X and 4X concentration. After a period of 10 days after subculture, various traits were assessed. The yellowing and wilting of the leaves were evaluated as qualitative traits and ranked accordingly. Additionally, leaf loss was documented based on the count of leaves detached in each treatment. The experimental was conducted a completely randomized design with three replications for each treatment.

Data Analysis

In this investigation, data analysis was conducted utilizing SAS version 9.1 software. The means comparison and statistical differences among treatments were analyzed through Duncan's multiple range test (DMRT) at both the one percent and five percent significance levels.

Results and discussion

Experiment 1- Effect of time, rootstock type, and culture medium injection on micrograft success

The analysis of variance results indicated that the timing of grafting did not significantly effect on scion growth characteristics, scion greenness, rootstock greenness, or callus formation. Furthermore, the type of rootstock significantly influenced scion growth at the %5 significance level, had a notable effect on callus development at the one percent level, but did not significantly affect scion greenness or rootstock greenness. Regarding the injection performed at the micrograft union, it did not significantly influence scion growth, scion greenness, or root greenness; however, it did significantly affect the quantity of callus produced at the micrograft union at the %1 level. Additionally, the interaction between time and rootstock type, as well as the interaction between time and injection, did not significantly affect any of the traits assessed in this study (scion growth, scion greenness, rootstock greenness, and callus). Conversely, the interaction between rootstock type and injection, along with the interaction among time, rootstock type, and injection, did not significantly impact scion growth, scion greenness, or root greenness, but did have a significant effect on callus formation at the %5 level (Table 1).

Table 1
Analysis of variance for the effects of time, rootstock type and medium injection on micrografting success

MS					
Source of variation	df	Scion growth	Scion greenness	Rootstock greenness	Callus induction
Time	2	0.08 ns	0.19 ns	0.07 ns	0.13 ns
Rootstock type	1	0.28 *	0.05 ns	0.12 ns	1.07 **
Injection	1	0.05 ns	0.01 ns	0.01 ns	1.61 **
Time × Rootstock	2	0.05 ns	0.01 ns	0.09 ns	0.09 ns
Time × Injection	2	0.14 ns	0.03 ns	0.03 ns	0.17 ns
Rootstock × Injection	1	0.12 ns	0.12 ns	0.04 ns	0.32 *
Time × Rootstock × Injection	2	0.07 ns	0.06 ns	0.10 ns	0.40 *
Error	24	0.05	0.05	0.03	0.07
CV (%)		10	11	8	24
** = significant at 1% level					
*= significant at 5% level					
ns = not significant at 1% or 5% levels					

In this experiment, the scion exhibited the highest growth rate when grafted onto the Japanese quince rootstock, achieving a rate of 2.2% (Fig. 2). Furthermore, the comparative analysis of the averages revealed that the highest callus formation rate occurred when the Japanese quince rootstock was carried out in late October and subsequently grafted onto the Pyracantha rootstock without the injection of the culture medium, resulting in a rate of %3. Conversely, the lowest callus formation rate, recorded at 0%, was noted at the end of the mid-October for plants grafted onto the Pyracantha rootstocks that received injections at the grafting union, as well as for those grafted onto the Japanese quince rootstocks that were injected at the micrografting union and those without any injection media (Table 2).

Based on the findings from the successful experiment, the rate of callus formation at the micrograft union under *in vitro* conditions was influenced by the time of the micrograft, the type of rootstock, and the injection media at the micrograft union. The impact of the rootstock on the growth rate of the scion has been validated by other researchers [29]. Soleimani et al. (2009) indicated that both the type of rootstock and the cultivar influence the grafting and growth of walnut scions. Their findings revealed that the Serr cultivar rootstock resulted in enhanced grafting and growth of the grafted seedling when compared to the Z63 cultivar [30]. The elevated percentage of grafting and growth associated with this rootstock is likely attributable to its superior traits, including enhanced growth vigor and more favorable

physiological quality. Furthermore, robust and rapidly growing rootstocks can significantly enhance grafting success in walnut trees [31]. In this study, the most significant growth of Japanese quince scions was recorded in the Pyracantha rootstock. This growth enhancement may be linked to improved rooting characteristics, increased water and nutrient transport, and more effective hormonal activities within the Pyracantha rootstock. Previous research has also indicated that high-growth rootstocks facilitate better sugar and hormone transfer to the scion, thereby promoting the vegetative growth of the scion [32, 33]. This phenomenon has led to variations in scion growth due to the physiological, anatomical, and biochemical disparities among different rootstocks. The rootstock can influence the growth and performance of the scion through factors such as nutrient absorption capacity, root system development, hormone secretion, and physiological interactions with the scion [34]. Additionally, Taghizadeh et al. (2025) noted that the type of rootstock significantly impacts the growth of scions grafted to Japanese quince under greenhouse conditions. They attributed this effect to the genetic differences present among the rootstocks [3]. The researchers attributed the observed effect to genetic variations among the rootstocks. The results of the study indicated that grafting enhances resistance to iron deficiency in Japanese quince, as employing an appropriate rootstock significantly improves the grafted plant's capacity to absorb and transport iron from the soil. Notably, Pyracantha rootstock demonstrated superior performance compared to other rootstocks in addressing iron chlorosis throughout the study. Japanese quince grafted onto Pyracantha rootstock exhibited greater vegetative growth and elevated chlorophyll content relative to other rootstocks. Furthermore, the analyses of iron and leaf pigments validated the efficacy of these techniques in detecting iron deficiency within the plant. Consequently, it is opinioned that Pyracantha rootstock represents a viable option for Japanese quince, as it enhances tolerance to iron chlorosis. Thus, the findings of this study align with the observations made by Taghizadeh et al. (2025) [3]. According to the result found from this experiment, the peak percentage of callus formation at the micrograft union was recorded in late October, particularly in Japanese quince micrografts on Pyracantha rootstock without the injection of culture medium at the graft union. The formation of callus at the grafting union represents the primary response of wound healing, which promotes adhesion between the scion and rootstock tissues, and is a precursor to the establishment of vascular continuity. The process of callus tissue development entails a coordinated sequence of cell division, dedifferentiation, and re-differentiation of both parenchymal and cambial cells. The timing, vigor, and cellular composition of the callus are significantly affected by the physiological condition of the plant material, as well as by external environmental factors that fluctuate with the seasons. It is crucial to comprehend how seasonal variables—such as temperature, photoperiod, water availability, carbohydrate reserves, and endogenous hormone levels—impact callus induction, as this knowledge is vital for optimizing grafting schedules and for formulating controlled propagation strategies that minimize seasonal dependence [35, 36].

The formation of callus at the graft union is regarded as an indicator of successful graft adhesion. Kamalzadeh et al. (2021) noted that for graft adhesion to be effective, callus formation must occur between the rootstock and the scion, with callus primarily originating from the basal tissue [37]. The repair and regeneration of vessels in micrografted plants constitute critical steps in achieving graft

success. During this process, parenchymal tissues regenerate from both the rootstock and scion, leading to the gradual formation of new xylem and phloem vessels at the graft union. The re-establishment of xylem vessels at the junction is particularly crucial, as these vessels are responsible for transporting raw sap from the roots to the aerial parts of the plant; without them, the scion cannot sustain its life effectively. Additionally, the coordination and compatibility between the rootstock and scion are essential for successful grafting. Furthermore, the interaction and compatibility between the rootstock and the scion are crucial for the development of new vessels at the grafting union [17]. Seasonal variation significantly impacts the physiological condition of donor plants and affects the success of grafting. Throughout the year, factors such as temperature, photoperiod, and levels of endogenous hormones fluctuate, influencing callus formation and the reconnection of vascular tissues at the graft union [38]. Temperature and metabolic rate – Elevated temperatures during the spring and early summer enhance cell division, auxin transport, and metabolic processes, thereby facilitating more rapid callus proliferation and earlier vascular reconnection. In contrast, lower temperatures experienced in winter impede mitosis and hormone movement, which can postpone callus formation and, in some cases, lead to increased necrosis at the cut surfaces [39]. Hormonal context (auxin, cytokinin, ethylene, jasmonate) – The levels of endogenous hormones vary with the seasons. The transport of auxin from the shoots to the graft union is crucial for the activation of the cambium and the vascular differentiation mediated by callus; cytokinin facilitates cell division. The seasonal variation of these hormones (such as reduced auxin flow during dormancy) influences the likelihood of forming wound callus and reconnecting vascular strands [35]. Research on woody species like *Malus domestica* and *Pyrus communis* indicates that explants collected during periods of active growth demonstrate higher rates of callogenesis and graft success in comparison to materials collected during the dormant season [40]. Nevertheless, there is a scarcity of studies that have thoroughly investigated this relationship in *Chaenomeles japonica*, especially under controlled *in vitro* conditions.

A noteworthy observation from this experiment is that during mid-to-early October, a greater amount of callus formation was noted at the grafting union in the treatment where the culture medium was not administered. However, by mid-November, the callus formation increased with the introduction of the culture medium into the grafting union. It can be inferred that until late October, when there was still a flow of cell sap in the phloem tissues of the mother rootstock, the explants possessed a higher reserve of nutrients and growth phytohormones (such as auxin). Sap flow represents the continuous movement of water and nutrients through the xylem and phloem tissues of vascular plants. During the cold season, this physiological process experiences substantial modifications due to environmental stresses such as freezing temperatures, reduced transpiration, and diminished soil water availability [41].

Consequently, the presence of IBA and nutrient compounds from the culture medium at the grafting union inhibited callus induction. Nevertheless, with the abrupt fall in temperature and the commencement of the dormancy period for the mother plants in mid-November, the nutrient levels in the mother tissues (explants) diminished, and the injection of a nutrient-rich culture medium into the grafting union cells stimulated callus formation. Huang and Millikan (1980) discovered that covering the micrografting union is essential for stabilizing the grafting tissues and retaining moisture necessary for

callus formation between the rootstock and scion [28]. Additionally, Pathirana and McKenzie (2005) indicated that the success rate of grafting improved when nutrient-saturated vermiculite was applied at the grafting union in grafted grapes [42]. The presence of a nutrient solution at the grafting union supplied water, moisture, and nutrients to the tissue at the junction of the rootstock and scion, leading to enhanced callus formation and the connection of the vascular bundles of both the rootstock and scion. Soheilivand et al. (2018) utilized MS medium containing 6 g L^{-1} of agar to sustain the grafting union and nourish the graft tissue [43]. Moreover, to prevent desiccation of the grafting union, MS medium with agar was injected every few days. Furthermore, to avoid desiccation of the grafting union, MS medium with agar was administered every few days. The findings indicated that the injection of nutrient medium at the micrografting union enhanced graft adhesion in lemon plants. These findings align with the outcomes of this experiment conducted in mid-November. Each graft type is executed only during specific periods of the year. Babaei et al. (2015) noted that both the rootstock type and the grafting timing significantly influence the propagation of *Ficus benjamina* [44]. Their findings revealed that grafting *Ficus benjamina* onto green rootstocks of the same species during summer yielded the highest rates of grafting and rooting. Additionally, the optimal grafting period for mango trees was identified as summer [45]. Moreover, Khajeh Ali and Mohammad Khani (2015) reported that the ideal months for walnut grafting, specifically for abdominal and patch grafting, were July and June, with the lowest grafting success occurring during the dormant season [46]. Sharma et al. (2022) indicated that the most favorable time for grafting walnut cuttings was in April on seed rootstocks [47].

Table 2
Effect of interaction effects of time, rootstock type and injection on micrografting success of Japanese quince

Time	Rootstock	Injection	Callus formation (coded) †
mid-October	Pyracantha	Injection	0.66 [e]
		Without injection	2.33 [b]
	Japanese quince	Injection	0.33 [f]
		Without injection	1.33 [c]
late October	Pyracantha	Injection	0 [g]
		Without injection	3 [a]
	Japanese quince	Injection	0 [g]
		Without injection	0 [g]
mid-November	Pyracantha	Injection	1 [d]
		Without injection	1 [d]
	Japanese quince	Injection	0.33 [f]
		Without injection	1 [d]

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

Experiment 2- Eliminating the effects of leaf senescence during the subculture of micrografted plants

Following grafting with the previous experimental protocols, adverse effects such as yellowing, wilting, and leaf fall inflicted significant harm on the micrografted plants prior to their adaptation phase, which was the focus of this experiment aimed at resolving this issue. The results demonstrated that micrografted plants treated with 10 mg L^{-1} silver nitrate in the culture medium exhibited no signs of yellowing, wilting, or leaf fall, marking a significant improvement over other treatments. An increase in iron concentration by fourfold compared to the control resulted in reduced leaf yellowing; however, wilting and leaf fall persisted in this treatment. The addition of vitamin C also failed to yield favorable outcomes in diminishing yellowing, wilting, and leaf fall when compared to the control. (Figs. 3, 4 and 5).

Micrografted plants experienced significant damage due to undesirable symptoms such as leaf yellowing, wilting, and leaf fall, prior to reaching the acclimatization phase, after the establishment of

grafts utilizing the treatments from the preceding experiment. In this study, a range of compounds was evaluated to address these issues. The findings revealed that micrografted plants treated with 10 mg L^{-1} silver nitrate ($AgNO_3$) exhibited no signs of chlorosis, wilting, or leaf abscission, a result that was markedly different from those of the other treatments. Although increasing the iron concentration to four times that of the control diminished leaf yellowing, wilting and leaf abscission persisted. Furthermore, the incorporation of vitamin C (ascorbic acid) did not yield a significant reduction in chlorosis, wilting, or leaf abscission when compared to the control (Fig. 3–5). Leaf yellowing and leaf fall are frequent physiological issues encountered during *in vitro* culture and acclimatization of plants. These manifestations are mainly linked to the accumulation of ethylene, which adversely impacts tissue differentiation and the overall vigor of the plant [48]. Silver nitrate ($AgNO_3$) is widely acknowledged as an effective inhibitor of ethylene action, thus enhancing the stability and vitality of regenerated shoots. In the last ten years, $AgNO_3$ has been incorporated into numerous micropropagation protocols for both ornamental and crop species to avert premature senescence and improve morphogenetic efficiency [49]. The primary mechanism of silver nitrate involves the release of Ag^+ ions, which competitively bind to ethylene receptors in plant tissues. This interaction inhibits ethylene from activating its downstream signaling pathway that leads to chlorophyll degradation and leaf abscission. By obstructing the ethylene perception pathway, $AgNO_3$ preserves the stability of photosynthetic pigments and postpones senescence [50]. Additionally, silver ions influence antioxidant enzyme activity, mitigating the accumulation of reactive oxygen species (ROS) under *in vitro* stress conditions, thereby enhancing tissue longevity. In numerous ornamental species, managing ethylene accumulation through the use of $AgNO_3$ or $CoCl_2$ represents the most effective strategy to reduce post-subculture disorders [51, 52]. In this study, the application of 10 mg L^{-1} $AgNO_3$ completely inhibited leaf yellowing, wilting, and leaf fall. $AgNO_3$ serves as a potent ethylene inhibitor, primarily through Ag^+ ions that deactivate ethylene receptors. The accumulation of ethylene under stress and wounding conditions generally induces chlorosis, necrosis, and abscission in plantlets. In rose micropropagation, AgNO3 at concentrations of 1.0–2.0 mg L^{-1} effectively mitigated physiological disorders like early leaf senescence and low multiplication rates caused by ethylene accumulation. Additionally, AgNO3 stimulated *in vitro* flowering induction, with 50% shoot flowering observed at 2.0 mg L^{-1}, although higher concentrations led to imperfect flower development [53]. Recent research indicates that the addition of $AgNO_3$ to the culture medium significantly enhances survival and photosynthetic stability in tissue-cultured plants [54, 55]. The inhibition of ethylene likely contributed to the stabilization of chlorophyll and enhanced water equilibrium within plant tissues. Although, increasing the concentration of Fe to four times that of the control diminished chlorosis but did not avert wilting or leaf abscission. Iron is crucial for the biosynthesis of chlorophyll, thereby directly mitigating leaf yellowing. Nevertheless, if root functionality or water transport is compromised, Fe alone is insufficient to prevent wilting. Recent investigations demonstrate that Fe supplementation is beneficial only when paired with stress management or moisture regulation [56]. Fe significantly enhanced chlorophyll levels but failed to alleviate physiological stress indicators. The addition of vitamin C to the media had no notable impact on chlorosis, wilting, or leaf fall. Ascorbate is recognized as a prominent cellular antioxidant; however, it exhibits instability under culture conditions, particularly when exposed to light. Recent research suggests that vitamin C is effective solely when

stabilized with agents such as cysteine or PVP [57]. The instability or inadequate concentration of ascorbic acid likely curtailed its efficacy.

Conclusions

The findings of this research indicated that the highest of callus formation was observed in late October, coinciding with the onset of the plant's physiological dormancy. This observation underscores the significance of employing tissue culture and micrografting techniques to extend the optimal grafting period. The findings of this experiment demonstrated that the type of rootstock proved a critical determinant of micrograft success. Rootstock type significantly affected both scion growth and callus formation, highlighting its pivotal role in graft compatibility and post-graft tissue regeneration. Likewise, the injection of culture medium, although not influential on growth or greenness attributes, exerted a significant impact on callus formation at the graft interface, indicating a potential physiological or biochemical modulation of wound-healing processes. Furthermore, significant interaction effects between rootstock type and injection treatments suggested that callus development is contingent upon combined factors rather than single variables alone. Nevertheless, in the current experiments, the implementation of sterile conditions, temperature regulation, and the selection of suitable culture media have mitigated seasonal limitations. Indeed, in contrast to field grafts that are influenced by ambient temperature, relative humidity, and the plant's physiological state, the tissue culture medium has enabled the induction of callus formation even during the dormancy phase. Favorable climatic conditions, including suitable temperature and relative humidity, facilitate the rapid establishment of vascular tissue connections between the rootstock and the scion. Consequently, outdoor grafting activities should be conducted during periods characterized by favorable temperature and relative humidity, when the vascular cambium is actively functioning. In contrast, micrografting does not impose such constraints on the grafting of plants. Also, Silver nitrate (10 mg L^{-1}) is the most effective treatment for eliminating all post-subculture physiological disorders in micrografted *Chaenomeles japonica*. Increasing Fe concentration reduces chlorosis but does not eliminate wilting or leaf fall and Vitamin C alone is not significantly effective. Future studies may test lower concentrations of $AgNO_3$ (5–10 mg L^{-1}) in combination with more stable antioxidants or ethylene-absorbing agents to further enhance plant survival and acclimatization. For future research, it is recommended to clarify the fundamental physiological and molecular mechanisms that dictate the variations in callus formation among different rootstock types, with a particular emphasis on hormone signaling and metabolic pathways that influence tissue union. Moreover, extending the temporal range of grafting periods and incorporating histological and biochemical evaluations could provide more profound insights into graft compatibility and the processes of vascular reconnection. Additionally, conducting experiments that encompass a wider genetic diversity among rootstocks and scions would assist in identifying genotypes that exhibit enhanced graft success, thereby aiding in the optimization of micrografting protocols within the field of horticultural biotechnology.

Abbreviations

AC: Activated Charcoal

$AgNO_3$: Silver Nitrate

MS: Murashige and Skoog

Vit C: Vitamin C

IBA: Indole Butyric Acid

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

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

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Figures

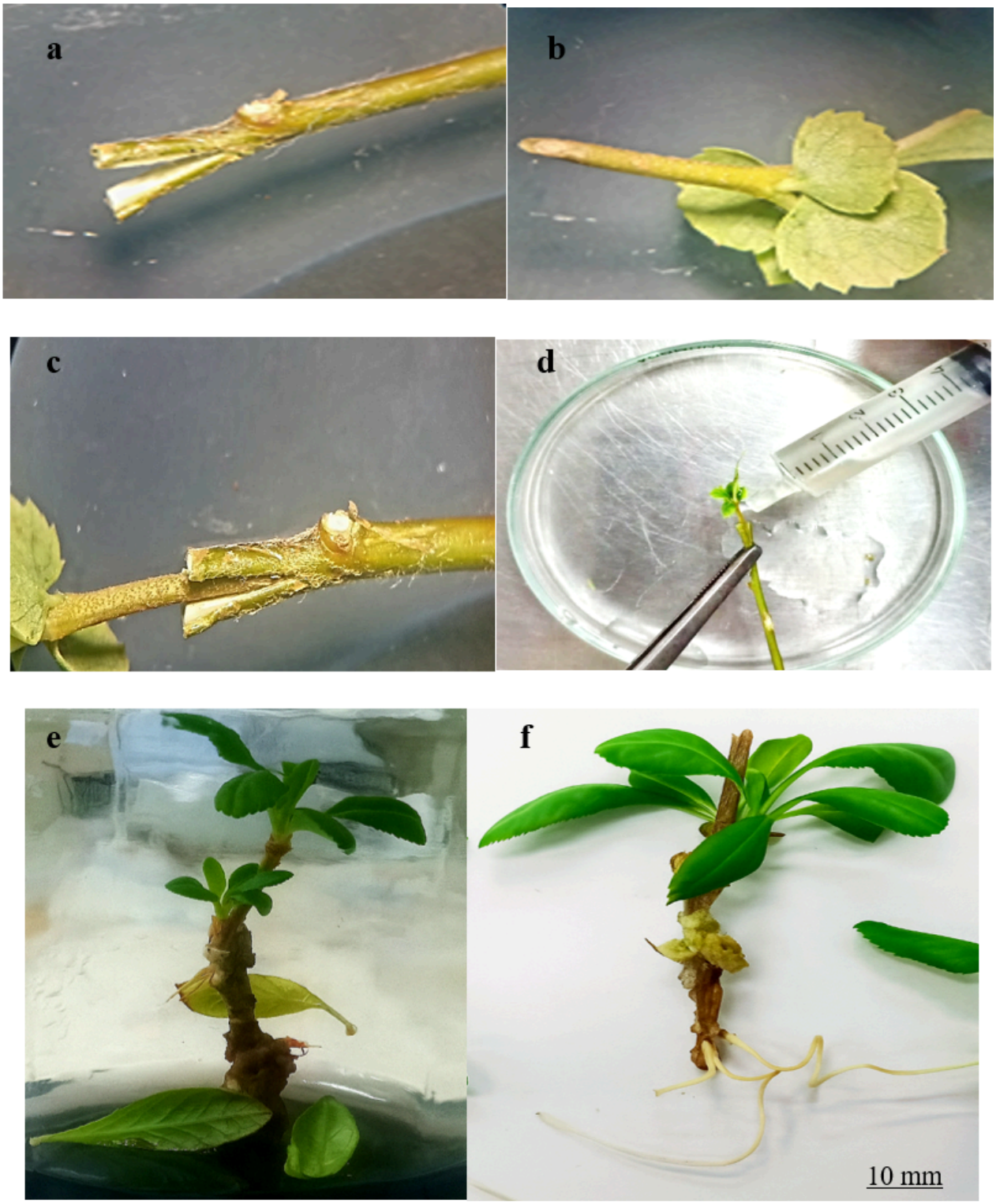


Figure 1

Performing cleft micrografting: preparation of pyracantha rootstock (a), preparation of Japanese quince scion (b), placement of scion in rootstock gap (c), injection of sterile culture medium into graft union (d), leaf shedding of micrografted plants during subculture (e), and full growth of Japanese quince micrograft on pyracantha rootstock after 8 weeks (f)

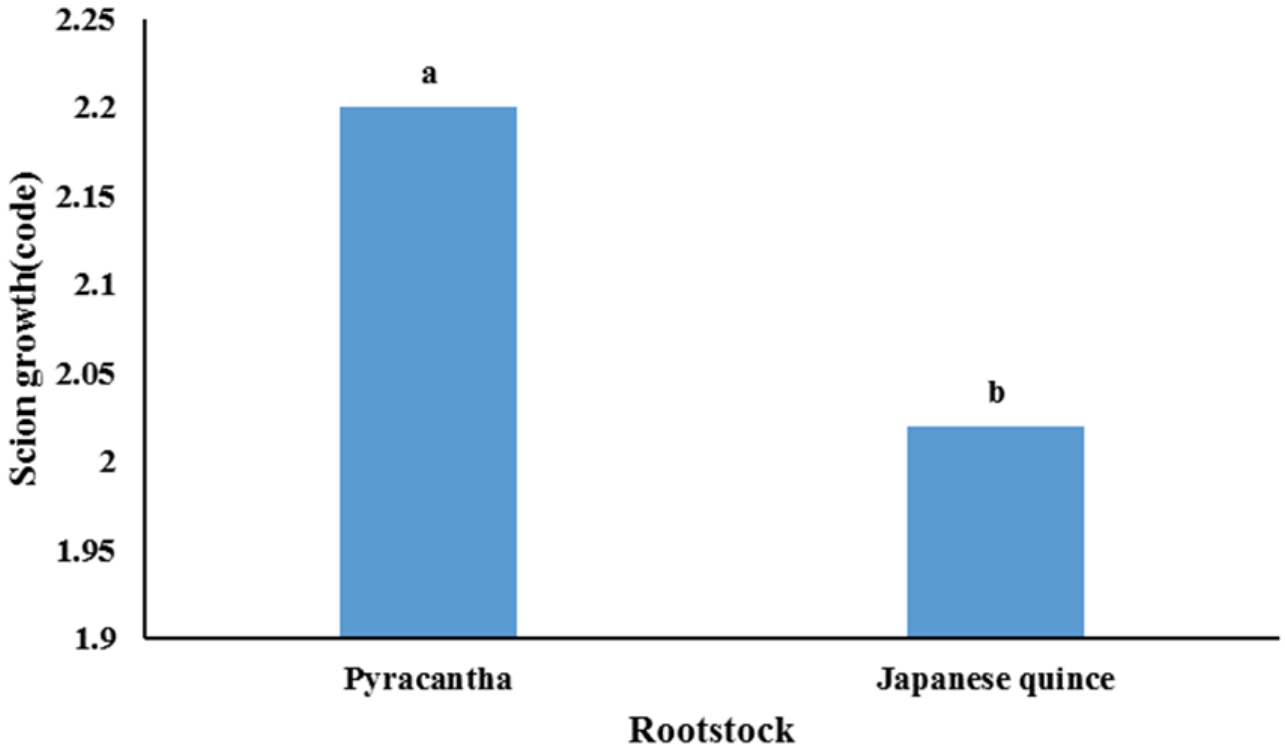


Figure 2

Effect of rootstock type on scion growth rate in micrografted plants

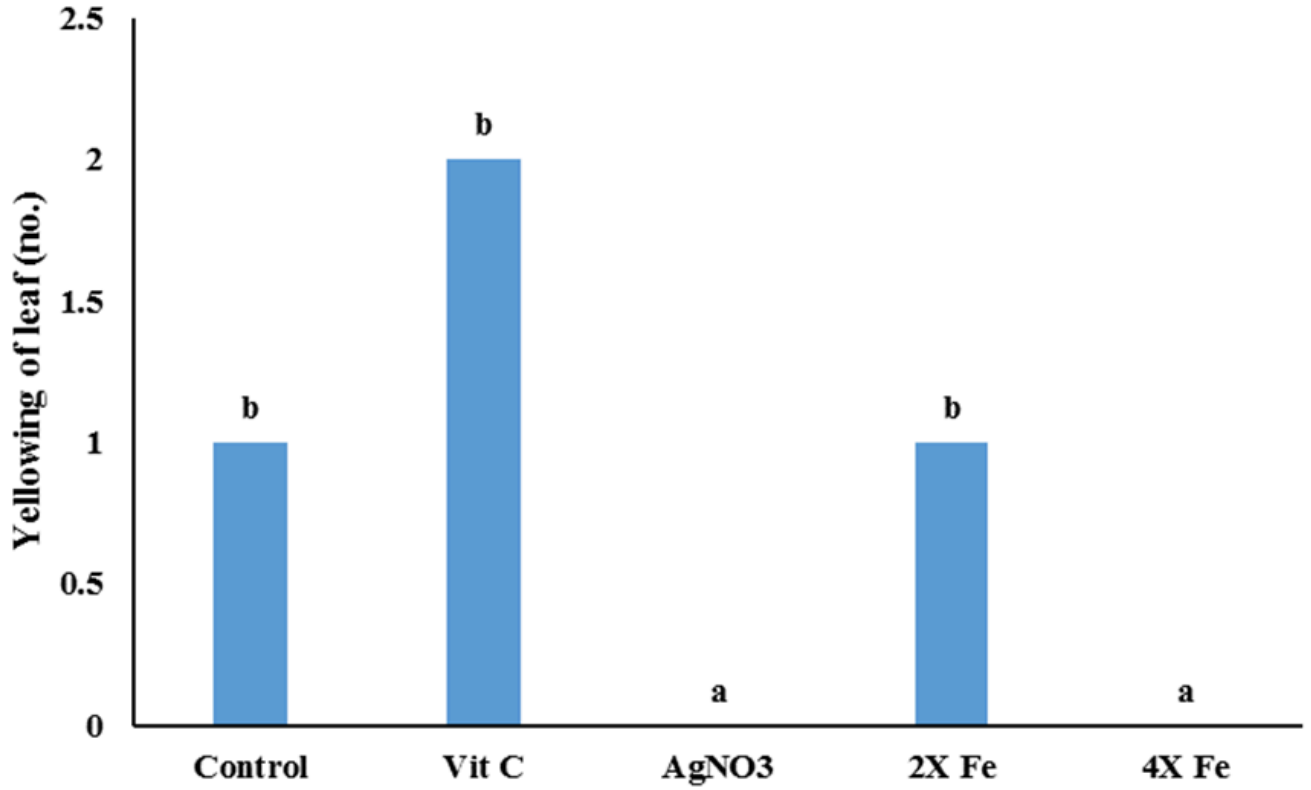


Figure 3

Effect of different compounds on leaf yellowing symptoms during subculture of micrografted plants

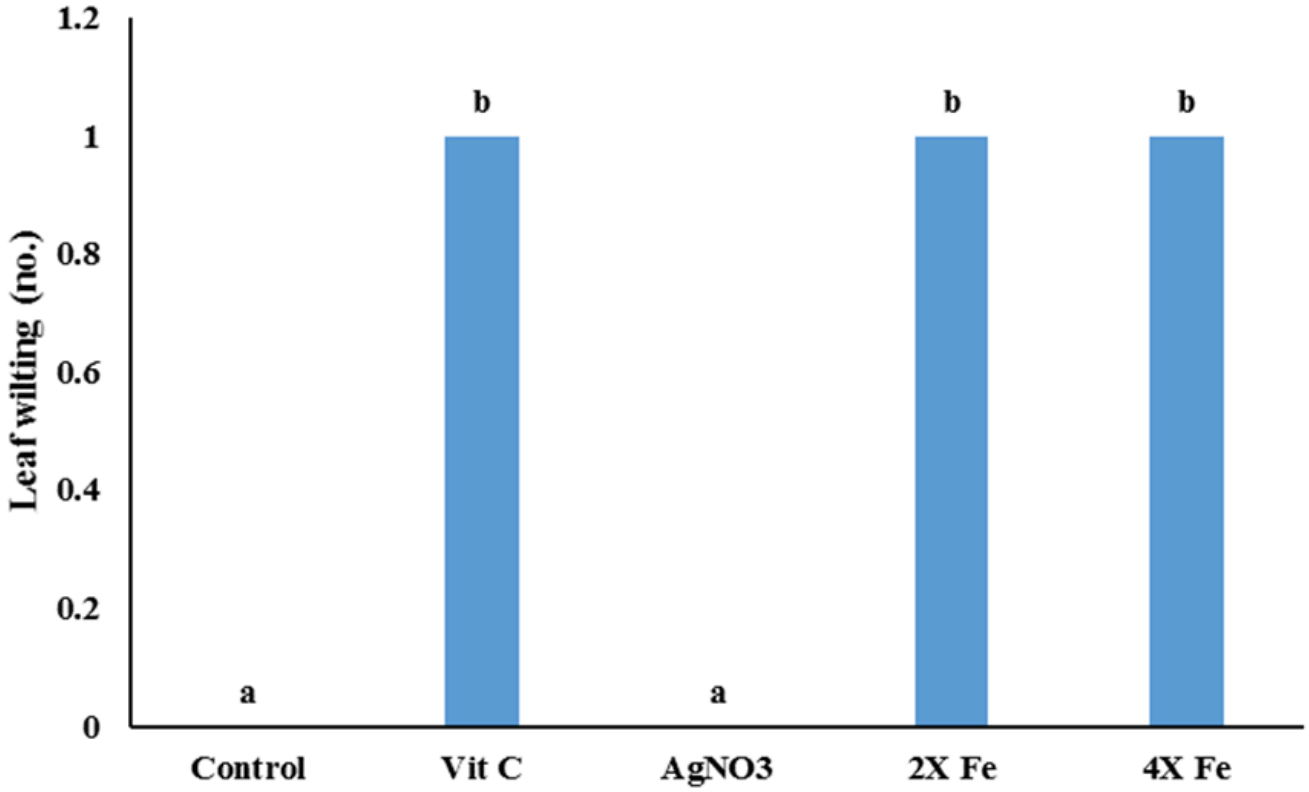


Figure 4

Effect of different compounds on leaf wilt symptoms during subculture of micrografted plants

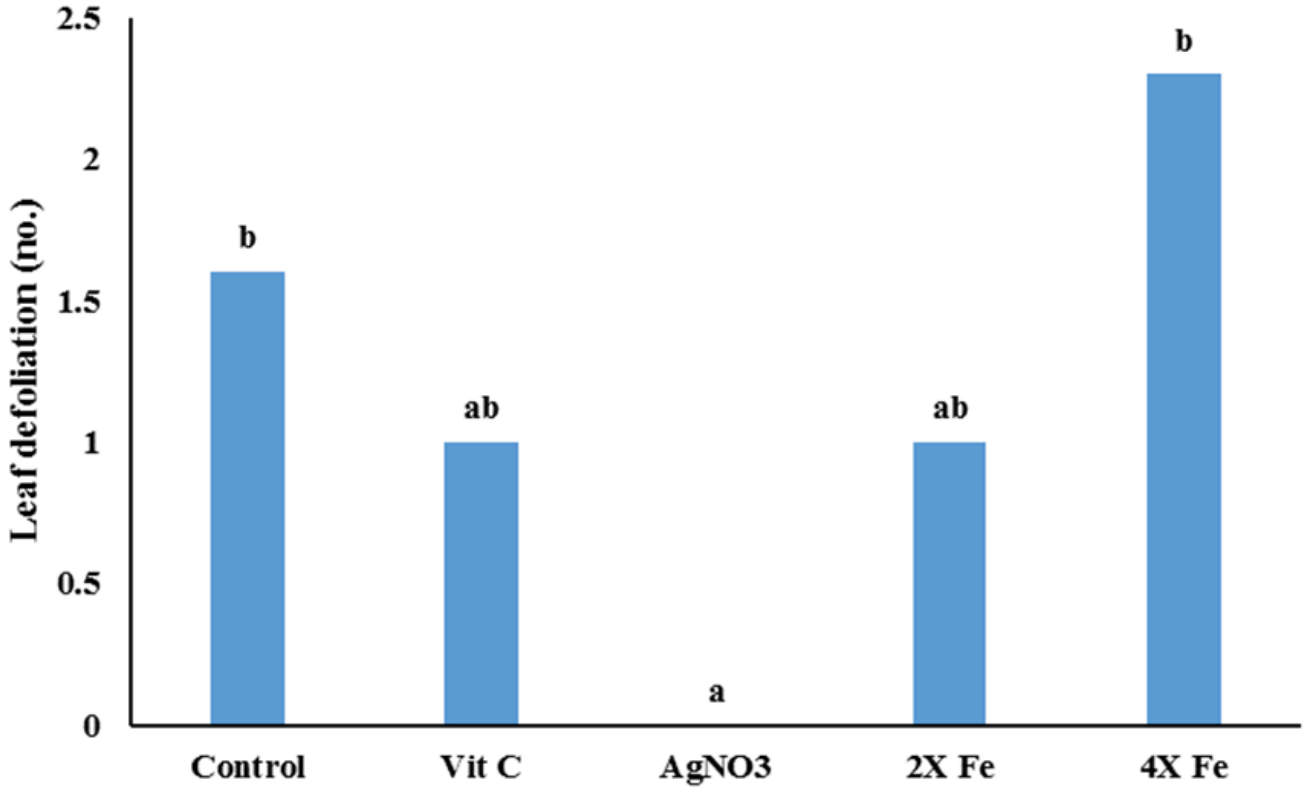


Figure 5

Effect of different compounds on leaf fall during subculture of micrografted plants