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THE EFFECTS OF SEVERAL VARIABLES ON SHOOT-TIP GRAFTING OF "CLEMENTINE" MANDARIN ONTO "TROYER" CITRANGE

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ABSTRACT

"Clementine" mandarin was successfully micrografted onto "Troyer" citrange [*Citrus sinensis* Osbeck × *Poncirus trifoliata* (L.) Raf.] rootstock *in vitro*. Shoot-tip grafted plants were transferred from culture tubes to greenhouse conditions by regrafting onto rough lemon seedlings. The effects of different closures of culture tubes on growth of seedlings were examined. The results showed that "Troyer" seedlings in culture tubes with rubber caps did not grow after seed germination, while seed germination and seedling growth with other closures took place normally. The effects of several variables on the micrografting success were also evaluated. When different concentrations of sodium hypochlorite were examined for shoot-tip sterilization, the best result was obtained with the concentration of 0.1% of this sterilizer. Pre-treatment of scions by different concentrations of 2,4-D and kinetin did not affect the grafting success rate. Similarly, the application of different concentrations of GA_3 or kinetin on quiescent scion tips did not induce seedling elongation. The highest rate of success (70%) was obtained when the concentrations of thiamine-HCl, pyridoxin-HCl and nicotinic acid were 14 times that of their recommended concentrations. The presence of light for scion survival was necessary and the lowest rate of lost scions (18%) was observed under 1500 lux illumination by cool white fluorescent lamps.

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اثر چند متغیر بر ریزپیوندی نوک شاخساره‌ای نارنگی «کلمانتین» روی پایه «ترویرسیترنج»

علیرضا شهسوار و مرتضی خوشخوی

به ترتیب دانشجوی کارشناسی ارشد (درحال حاضر مربی) و استاد بخش باغبانی دانشکده کشاورزی، دانشگاه شیراز، شیراز، ایران.

چکیده

ریزپیوندی نارنگی «کلمانتین» روی پایه «ترویرسیترنج» با موفقیت انجام گرفت و با پیوند مجدد گیاهان پیوند زده شده روی پایه راف‌لمون، انتقال گیاهانی که با ریزپیوندی افزوده شده بودند از لوله‌های کشت به شرایط گلخانه با موفقیت امکان‌پذیر گردید. اثر درپوشهای مختلف لوله‌های کشت بر روی رشد دانهال‌ها نیز مورد آزمایش قرار گرفت. نتایج حاصله نشان داد بذرهایی که در لوله‌های کشت با درپوش لاستیکی کاشته شده بودند، جوانه زدند ولی رشد نکردند، اما بذرهایی که در سایر تیمارها کاشته شده بودند، جوانه زدند و بطور طبیعی رشد کردند. در این پژوهش اثر چند عامل بر روی موفقیت پیوند نیز مورد بررسی قرار گرفت. غلظتهای مختلف هیپوکلریت سدیم جهت گندزدایی نوک شاخساره‌ها مورد بررسی قرار گرفت و بهترین نتیجه در غلظت ۰/۱ درصد بدست آمد. قرار دادن پیوندک در غلظتهای مختلف 2,4-D و کینتین، پیش از عمل پیوند، اثری در افزایش میزان پیوندهای موفق نداشت. کاربرد غلظتهای مختلف GA_3 و کینتین در تحریک رشد پیوندک‌هایی که در محل پیوند، زنده می‌ماندند ولی رشد نمی کردند، اثری نداشت. بالاترین میزان موفقیت پیوند (۷۰٪) وقتی بدست آمد که ویتامینهای هیدروکلرید تیامین، هیدروکلرید پیریدوکسین و اسید نیکوتینیک با ۱۴ برابر غلظت توصیه شده در محیط کشت بکار رفتند. وجود نور برای بقای پیوندک لازم تشخیص داده شد. بهترین میزان شدت نور ۱۵۰۰ لوکس بود که در این شرایط نوری، میزان پیوندک‌های از دست رفته در مقایسه با سایر تیمارها کمترین یعنی ۱۸٪ بود.

INTRODUCTION

Citrus crops are the most important subtropical fruits in the world. One of the most important limiting factors in citrus production, is the damage caused by virus and virus-like diseases. Although thermotherapy has been employed to provide budwood free of certain viruses (2, 11, 13), it has been an ineffective method for eliminating certain viruses (2, 11, 16, 17). Meristem cultures have been used successfully for production of virus-free herbaceous crops, but this technique is not yet applicable to woody genera (5, 7, 8).

In the Rutaceae family, plants arising from embryogenesis of the nucellus have been shown to be free of most pathogenic viruses (1, 12). Unfortunately, such plants show juvenile state (10). Murashige *et al.* (7) suggested the method of *in vitro* shoot-tip grafting and this method can be used to obtain citrus plants free of all known virus and virus-like diseases without having juvenile characteristics (9, 10, 14, 15).

The purpose of the present study was to micrograft "Clementine" mandarin as scion on "Troyer" citrange seedlings as rootstock, to transfer the micrografted plants from culture tubes to greenhouse conditions and to evaluate the effects of several variables that might influence micrografting success.

MATERIALS AND METHODS

The methods used by Navarro *et al.* (10) were followed for stock preparation, scion preparation and grafting procedures.

Stock Preparation

"Troyer" citrange [*Citrus sinensis* Osbeck × *Poncirus trifoliata* (L.) Raf.] was selected as the stock. The seeds were washed by water, peeled by removing both seed coats, wrapped in cheesecloth and

surface-sterilized by immersion in a 0.5% sodium hypochlorite, plus 0.1% Tween-20 for 10 min and rinsed 3 times with autoclaved distilled water. The germination medium was Murashige and Skoog major salts (6), solidified with 1% Difco Bacto-agar. The pH of the medium was initially set at 5.7±0.1. The medium was distributed in 20-ml aliquots in 25×150 mm culture tubes and sterilized by autoclaving at 121°C and 1.5 kg cm $^{-2}$ pressure for 15 min. One seed was sown per tube and allowed to germinate at a constant temperature of 27°C in continuous darkness for two weeks. To investigate the effects of different culture tube closures on growth of seedlings a completely randomized design with 4 treatments and 12 replications was conducted. The treatments consisted of: a) culture tubes with screw caps as control; b) culture tubes with rubber caps wrapped in a piece of aluminum foil; c) culture tubes with screw caps, plus a piece of rubber cap inside the tube, and d) culture tubes with rubber caps. After 15 days the length of seedlings from shoot-tip to root-tip was measured. The data were analyzed and the means compared by Duncan's multiple range test.

Scion Preparation

The shoot-tips were obtained from actively growing new shoots on greenhouse-grown "Clementine" mandarin (*Citrus reticulata* Blanco) plants. To induce flushing, plants were completely defoliated by hand and kept in a warm greenhouse (24 to 32°C). Depending on greenhouse temperature many buds were produced after 10 to 15 days (Fig. 1). Flushes 3 cm long or less were chosen, their larger leaves stripped, wrapped in small pieces of cheesecloth and surface sterilized by soaking in 0.25% sodium hypochlorite, plus 0.1% Tween-20 for 5 min. The sterilized tissues were rinsed 3 times with autoclaved distilled water and their shoot tips were excised and used as scions.

Since most of the scions were burned and dried several days after micrografting, lower concentrations of sodium hypochlorite were

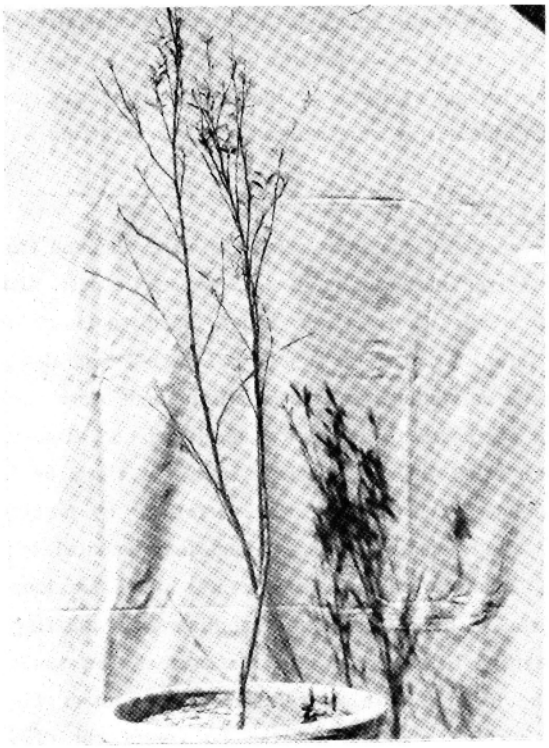

Fig. 1. A greenhouse-grown "Clementine" mandarin tree, 13 days after defoliation.

examined. The concentrations used were 0.05, 0.1, 0.15 and 0.25% . Shoot-tips were soaked in these solutions for 5 min and were rinsed 3 times with autoclaved distilled water.

Pre-treatments of scions with growth regulators were accomplished by dipping the shoot apices in different concentrations of 2,4-D (6, 8, 10, 12 mg l^{-1}) and or in kinetin (0.1, 0.5, 1, 1.5 mg l^{-1}) each for 6 and 10 min prior to excision of the apical dome and primordial leaves. After that, shoot-tips were excised and micrografting was performed.

Grafting Procedure

Two-week old rootstock seedlings were removed from the test tubes and transferred individually to a 15-cm sterile petri dish lined with one sheet of sterile, moist filter paper. Each rootstock was decapitated, leaving 1 to 1.5 cm of the epicotyl. The root was also shortened to 4-6 cm and the cotyledons and their axillary buds were removed (Fig. 2). A shoot-tip composed of the apical meristem and adjacent tissues with 3 to 4 leaf primordia was excised from the flush by a scalpel with a No. 11 blade. Micrografting was performed by making an inverted-T incision. The incisions were a 1-mm long vertical cut starting at the point of decapitation and a horizontal cut 1 mm long. The cuts were made through the cortex to the cambium, and the flaps of the incision were slightly lifted to expose the cortex. The shoot-tip was placed inside the incision with its cut surface in contact with the roots'ock's cortical surface, exposed by the horizontal cut of incision. Grafted plants were aseptically cultured in a liquid medium containing the MS major salts (6) plus 0.2 mg l^{-1} thiamine-HCl, 1.0 mg l^{-1} pyridoxin-HCl, 1.0 mg l^{-1} nicotinic acid, 100 mg l^{-1} i-inositol and 75 g l^{-1} sucrose (9). The pH of the solution was set at 5.7±0.1 prior to autoclaving. The medium was distributed into 25×150 mm culture tubes in 20 ml aliquots. A supportive platform, made from a folded 9 cm circle of Whatman filter paper, was placed in the nutrient solution. The culture tubes

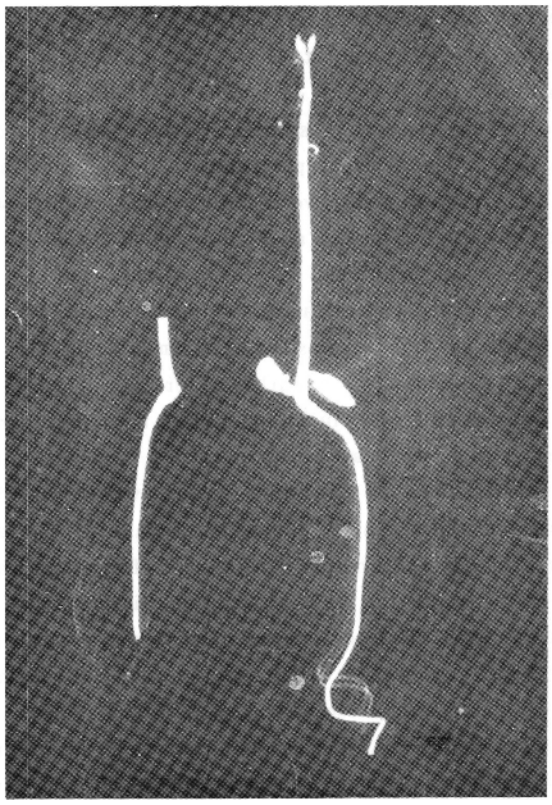

Fig. 2. The procedure of rootsdtock preparation. Right: a 2-week old "Troyer" citrange seedling; Left: a seedling rootstock prior to grafting with epicotyl decapitated, cotyledon and root-tip severed.

containing medium were sterilized by autoclaving at 121°C temperature and 1.5 kg cm^{-2} pressure for 15 min. The grafted plants were transferred to culture tubes at 27±3°C and exposed to 16-hr daily of 1500 lux illumination, provided by cool white fluorescent lamps.

For increasing the rate of successful grafts, different concentrations of B-vitamins, thiamine-HCl, pyridoxin-HCl, and nicotinic acid were evaluated. A group of plants were cultured in a medium with a normal concentration of B-vitamins (0.2 mg l^{-1} thiamine-HCl, 1 mg l^{-1} pyridoxin-HCl, and 1 mg l^{-1} nicotinic acid), and the other groups in media with 2 to 20 times of recommended concentrations of these vitamins (10).

The following light intensity treatments were also tested: a) A group of plants were kept in darkness for 5 days after micrografting and then transferred to 800 lux illumination; b) Another group was kept in darkness for 5 days after micrografting and then transferred to 1500 lux illumination; c) The third group was exposed to 800 lux illumination immediately after micrografting; and d) The last group was exposed to 1500 lux illumination immediately after micrografting.

Some scion shoot-tips were alive after micrografting but remained in a quiescent state for several months. To induce growth, a drop of 20, 40 or 60 mg l^{-1} gibberellic acid (GA_3) or 1, 3 or 5 mg l^{-1} kinetin solution was placed onto these quiescent shoot-tips.

For transferring the micrografted plants from culture tubes to greenhouse conditions, when the shoot-tip grafted plants were about 1 to 2 cm in size (Fig. 3-A), they were removed and grafted directly from the test tube onto well-established clean rough lemon (*Citrus jambhiri* Lush.) seedlings (about 10 mm in diameter) in the greenhouse (3). The shoot-tip grafted plant was sliced behind the graft so that there was a flat area to make contact with the rough lemon cambial tissue (Fig. 3-B). The leaves were detached (Fig. 3-C) and a slice of shoot-tip grafted plant was placed into T-cut and wrappped with budding tape (Fig. 4). After acclimatization, the growth of the grafted plant thereafter continued under a conventional greenhouse condition.

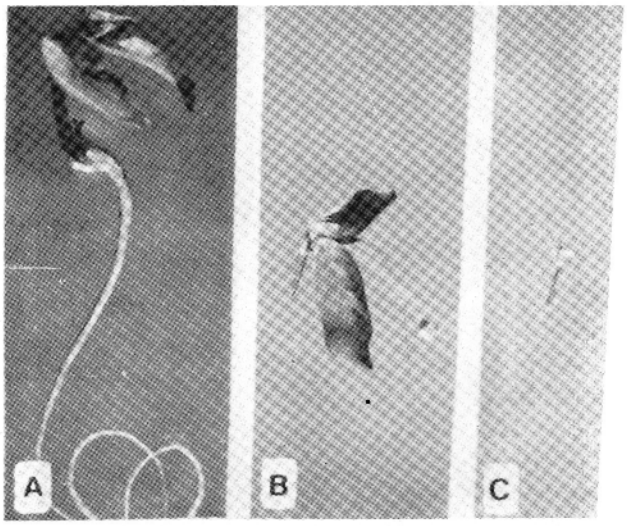

Fig. 3. Preparation of shoot-tip grafted plant for regrafting. A: Shoot-tip grafted "Clementine" mandarin on "Troyer" citrange rootstock taken from a test tube; B: The grafted plant sliced behind the graft union; C: The grafted union with the leaves detached.

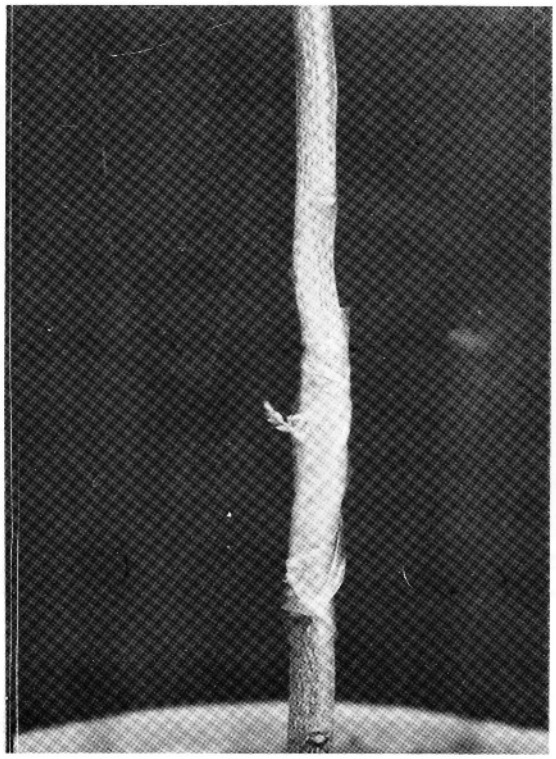

Fig. 4. Regrafting of shoot tip grafted plant on the rough lemon seedling.

RESULTS AND DISCUSSION

In general, rate of shoot-tip grafting of "Clementine" mandarin onto "Troyer" citrange rootstock was between 40 to 45% . Shoot-tip grafted plants were successfully transferred from culture tubes to greenhouse conditions by grafting them onto rough lemon seedlings.

Effects of Culture Tube Closures

Different culture tube closures showed significantly different effects on growth of seedlings. All seeds of the following treatments germinated and grew normally: a) culture tubes with screw caps; b) culture tubes with rubber caps wrapped in aluminum foil; and c) culture tubes with screw caps plus a piece of a rubber cap inside the tube (Fig. 5). After 15 days, the seedlings in these treatments were ready for grafting procedure. No significant differences were obtained among these treatments (Table 1). However, a highly significant differences were obtained among the growth of seedlings in culture tubes with rubber caps and other treatments. The seeds in culture tubes with rubber caps germinated but did not grow (Fig. 5-D). First, it was presumed that the black color of rubber caps was responsible for the lack of growth of seedlings in culture tubes covered with rubber caps, but when light color rubber caps were used, again the seeds did not grow normally, showing that no growth inhibition was induced by black color. Second, it was possible that rubber caps released some inhibitory materials into tube environment. Placing a piece of rubber cap in the culture tube did not inhibit the growth of seedlings showing that no inhibitory materials were involved. Finally, it was concluded that the lack of gaseous exchange between tube content and external environment and/or accumulation of harmful gases such as CO_2 and

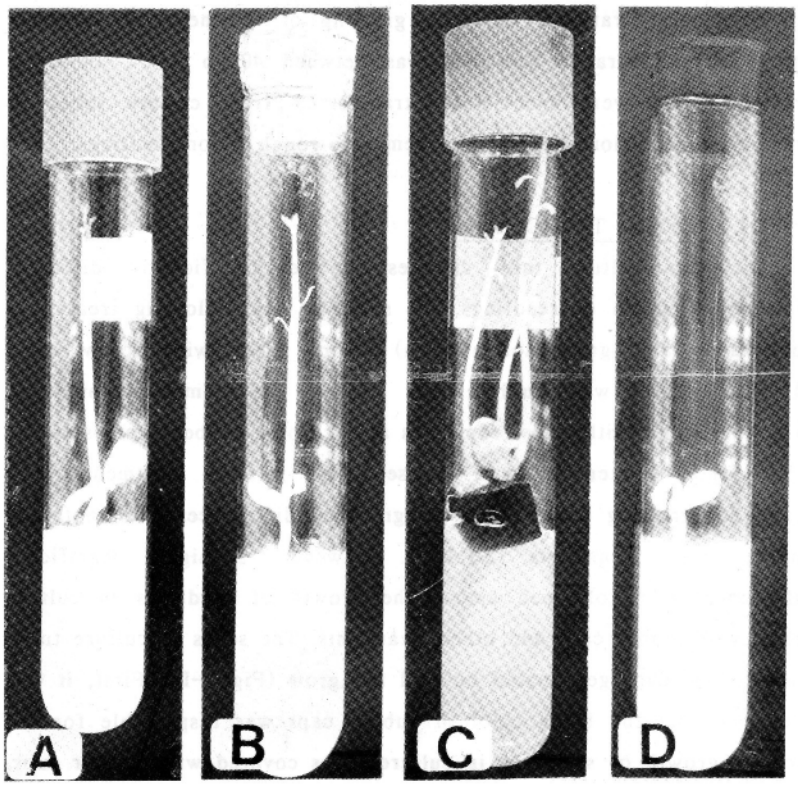

Fig. 5. Comparison of different culture tube closures. A: Culture tube with screw cap; B: Culture tube with rubber cap wrapped in aluminum foil; C: Culture tube with screw cap plus a piece of a rubber cap inside the tube; D: Culture tube with rubber cap.

ethylene and reduction of useful gases such as O_2, due to tight contact of rubber caps with culture tubes might be responsible for this growth inhibiting effect of rubber caps. Further experiments may be necessary to elucidate this point.

Table 1. Effect of different culture tube closures on growth of "Troyer" citrange seedlings.

Treatments	Mean plant length (cm)
Culture tubes with screw caps (control)	18.0 a†
Culture tubes with rubber caps wrapped in aluminum foil	17.1 a
Culture tubes with screw caps, plus a piece of rubber cap inside the tube	17.4 a
Culture tubes with rubber caps	3.3 b

† Means followed by the same letter are not significantly different at the 1% probability level, using Duncan's multiple range test.

Effects of Sodium Hypochlorite Concentrations

The highest percentage of successful grafts (40%) without any contamination was obtained with 0.1% sodium hypochlorite. This result was not in accordance with the data obtained by Navarro *et al.* (10) who proposed that 0.25% sodium hypochlorite solution was the best

concentration. In our investigation the latter concentration of sodium hypochlorite caused burning and destruction of scion shoot-tip tissues. Genotypic and/or environmental differences as well as impurities of the sodium hypochlorite source might be the reasons for such response.

Effects of Plant Growth Regulators

Using 6, 8, 10 and 12 mg l^{-1} of 2, 4-D and 0.1, 0.5, 1 and 1.5 mg l^{-1} of kinetin each for 6 and 10 min, as pre-treatments of scions, did not affect the micrograft success. Most of the shoot-tips did not survive and those which remained alive were quiescent. The maximum rate of successful grafts was only 13% . This observation is in contrast with the results obtained by Edriss and Burger (4) who found that dipping shoot-tips in 2,4-D (10 mg l^{-1}) or kinetin (1 mg l^{-1}) for 5-10 min, before grafting, doubled the success rate.

Effects of Plant Growth Regulators Applied to Quiescent Scion Tips

The application of 3 concentrations of GA_3 or kinetin solutions to quiescent scion tips failed to induce shoot-tip elongation. However, in some instances use of GA_3 solutions caused elongation of the adventitious buds. This observation confirms the results obtained by Navarro *et al.* (10).

Effects of Vitamins

The highest rate of successful micrografting (70%) was obtained when the concentrations of thiamine-HCl, pyridoxin-HCl, and nicotinic acid were 14 times of those recommended concentrations, while the rate of successful grafts in the control was 60% . This observation is in agreement with that of Navarro *et al.* (10), who found that application of these vitamins slightly increased grafting success.

Effects of Light

The presence of light for scion survival was necessary. When the grafted plants were placed under darkness for 5 days, and then transferred under 800 and 1500 lux illumination, the scions did not survive in 82% of cases. In plants placed under 800 lux illumination just after grafting, 73% of scions were lost. When the plants were placed under 1500 lux illumination only 18% of scions failed to survive (Fig. 6). This observation is in accordance with the results obtained by Navarro *et al.* (10).

CONCLUSION

On the basis of the data obtained in this investigation it might be concluded that using rubber caps are not recommended for culture tubes, since they inhibited the plant growth in micrografting. However, wrapping the caps in aluminum foil can overcome this problem. The optimum concentration of sodium hypochlorite for disnifection of shoot tips was 0.1%. Pre-treatment of scions by different concentrations of 2,4-D and kinetin did not affect the grafting success. Similarly, the application of different concentrations of GA_3 or kinetin on quiescent scion tips did not promote the scion growth. Application of high concentrations of vitamins slightly increased the grafting success. Light intensity of 1500 lux was necessary for growth of micrografted plants.

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L_1= 5 days in darkness, then transferring under 800 lux illumination.

L_2= 5 days in darkness, then transferring under 1,500 lux illumination.

L3= Under 800 lux illumination justafter grafting.

L_4= Under 1,500 lux illumination just after grafting.

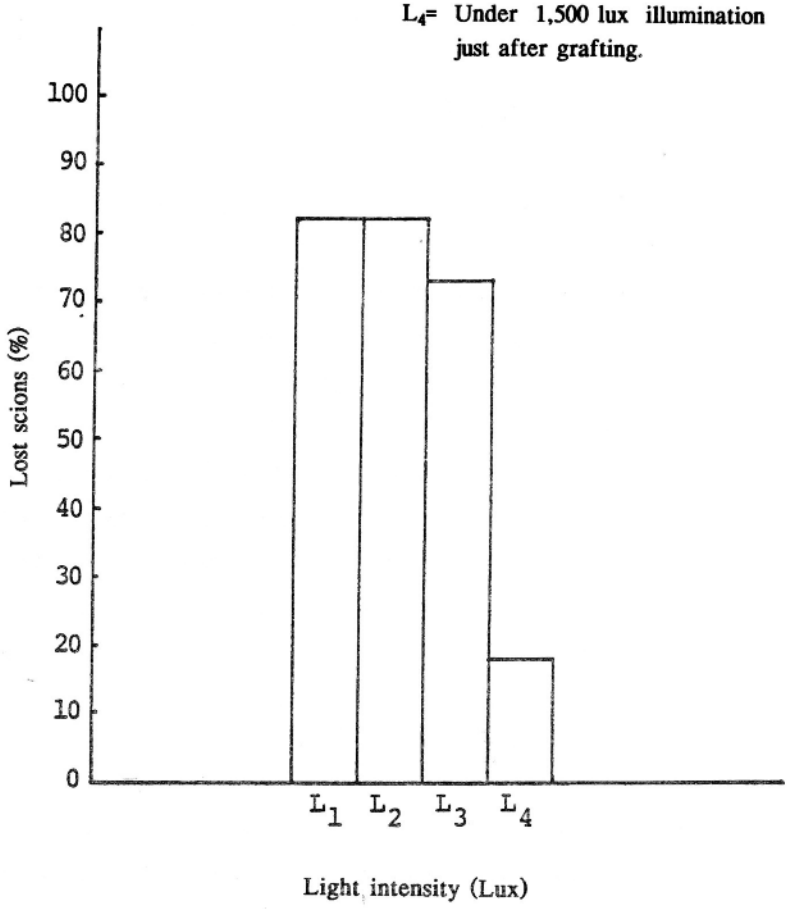

Fig. 6. The effects of different light treatments on scion survival.

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