Effects of Plant Growth Regulators on in Vitro Propagation of Trifoliate Orange©

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The rate of shoot multiplication and rooting capacity in the course of subculture and the effects of auxin and cytokinin combinations on shoot proliferation and rooting in vitro were determined by using trifoliate orange (*Poncirus trifoliata* [L] Raf.). Shoot development was high in second, third, and fourth generations, and gradually decreased after the fifth generation. The rooting percentage was highest in the second generation after 30 days of culture. Rooting gradually decreased from the third generation shoots and there was no rooting in the sixth generation shoots. Shoot and root number and length were higher in the second and third generations. Shoot number, shoot length, and callus formation were increased by BA, but BA did not induce rooting. For root and shoot development, the group of treatments where IAA or NAA was used alone or IBA and IAA were used in combinations gave the best results among the various groups.

INTRODUCTION

Trifoliate orange is an important rootstock for citrus in many parts of the world and is usually propagated from seed. However, since zygotic seedlings are present among nucellar ones, it is necessary to remove them to achieve rootstock uniformity. The frequency of zygotics detected ranges from 0% to 76% depending on cultivar and environmental factors (Khan and Roose, 1988). Trifoliate orange is the most tolerant of both flooding and drought among the several citrus species and cultivars (Bhusal et al., 2002a).

Tissue culture is a powerful tool for multiplying disease-free transplants and is a suitable technology for the propagation of fruit crops. In vitro culture has advantages because it is able to minimize undefined variables and carefully control medium composition and environmental factors (Harada et al. 2001). Tissue culture is rapidly becoming a commercial method for propagation to reduce production costs (especially for rootstocks that are difficult to propagate economically by standard practices); to satisfy a demand for clonal rootstocks that is becoming more and more specific; and to produce a great quantity of material in a small space, starting from a few mother plants (Fiorino and Loreti, 1987). Juvenility of stock plants is one of the most important factors affecting rooting success in cutting propagation of trifoliate orange. The ease of adventitious root formation declines with the age of the stock plant, resulting in a propagation problem in trifoliate orange (Bhusal et al., 2002b). In this context, in vitro propagation might be used to overcome this problem by producing stock plants with juvenile-like characteristics. Some potential citrus rootstocks produce a limited number of seeds and periodic seed shortage occurs. For this reason also, the use of tissue culture might be useful as an alternative propagation method. The first objective of the current investigation was to determine the rate of shoot multiplication and rooting capacity of different generations of

trifoliate orange in vitro. The second objective was to determine the effect of auxin and cytokinin combinations on shoot proliferation and rooting in trifoliate orange cultured in vitro.

MATERIALS AND METHODS

The experiments were conducted at the University Farm, Ehime University, Japan between November 2001 and August 2002. Seeds of trifoliate orange were obtained from matured fruit and washed several times with tap water. The outer and inner seed coat were removed from the seeds and the seeds were surface-disinfected with 10% sodium hypochlorite solution for 15 min. Then they were soaked in 70% ethanol for 30 sec. and rinsed about 20 times in sterile distilled water. The disinfected seeds were allowed to germinate and grow on 10 ml of 1.5% agar media in 25 × 120 mm test tubes for 2 months. For shoot development, MS salts (Murashige and Skoog, 1962) supplemented with BA (1 mg · liter^{-1}) and organic substances followed by a modified protocol of Starrantino and Caruso (1987) were employed (Table 1). On the other hand for the root formation, BA (1 mg · liter^{-1}) was replaced with IBA (1 mg · liter^{-1}) in the above medium. For obtaining efficient shoot development and root formation, various combinations of BA, IAA, IBA, and NAA were also tested at 1 mg · liter^{-1} for all the regulators. Explants were cultured in 25 × 120 mm test tubes, filled with 10 ml of medium. The pH of the media was adjusted to 5.7 with 1 N KOH prior to autoclaving. Single node explants were taken from the 2-month-old seedlings and transplanted into the above-mentioned media for rooting and shoot development. Shoots from every new generation were transplanted at monthly intervals up to the sixth generations. At least 30 explants were used per treatment at each stage of the culture. Rooting and shoot development potential along with root and shoot length were measured two times for each generation; that is 15 and 30 days after shoot transplanting. After completing the proliferation and rooting stage, the well-rooted explants plants were thoroughly washed with tap water to remove agar medium from the roots and transferred to plastic pots that contained vermiculite. The pots were placed in a tray that was covered with plastic to avoid desiccation. After 2 weeks, some holes were made in the plastic and it was gradually removed. After 1 month, pots were taken out from the temperature-controlled room and placed in a greenhouse with mist irrigation for hardening and acclimatization.

RESULTS AND DISCUSSION

Shoot development was observed in all generations but it was high in the second, third, and fourth generations, and gradually decreased after the fifth generation (Fig. 1). In the evaluation after 30-day culture, the rooting percentage was highest in the second followed by third generation shoots. Rooting gradually decreased from the third generation and there was no rooting in the sixth generation shoots (Fig. 2). Shoot and root number and length were higher in the second and third generations (data not presented). In the IAA, IBA, NAA, and BA combination experiments, shoots rooted only in the absence of BA. BA increased the shoot number, shoot length, and callus formation but induced no rooting. Root numbers were significantly higher in other auxin treatments than in the IBA treatment but in some combinations, no shoot was formed. For root and shoot development, the group of treatments where IAA or NAA was used alone or IBA and IAA were used in combinations gave the best results among the various groups (Table 2).

Table 1. Tissue culture steps and relative media employed.

Steps	Media
1) For seed germination	Agar 15,000 mg · liter^{-1}
2) For shoot development	MS salts + BA (1 mg · liter^{-1}) + organic substances (mg · liter^{-1}): myo-inositol (100) thiamine H Cl (0.4) pyridoxine HCl (0.5) nicotinic acid (0.5) glycine (2.0) adenine (25) sucrose (50,000) malt extract (500) agar (8000)
3) For rooting	MS salts + organic substances + IBA (1 mg · liter^{-1})
4) To know the better shoot and root development	MS salts + organic substances + different combinations of BA + IAA + IBA + NAA (1 mg·liter^{-1} for each regulator)

Bhansali and Arya (1978) reported that a higher concentration of cytokinins reduced callus growth and shoot development and that shoots formed were also unhealthy. In our preliminary experiments, neither shoot elongation nor root formation was enhanced by cytokinin or auxin concentrations above 2 mg · liter^{-1}. The best shoot and root formation occurred at a concentration of 1 mg · liter^{-1} for both cytokinin and auxin (data not presented). This concentration was used in all treatments in this experiment. According to Kotsias and Roussos (2001), when auxins were added to the medium, auxin type and combination affected rooting percentage and NAA gave the lowest rooting percentage of lemon in vitro. But Starrantino and Caruso (1988) reported that high rooting percentages have been achieved with other species by the addition of NAA to the medium. Harada and Murai (1996) mentioned in their report that the frequency of root formation of trifoliate orange on the medium supplemented with IBA was 90 to 100%. Thus, the preference for a specific auxin may vary from species to species and cultivar to cultivar. In our experiment, root and shoot development was highest when, IAA or NAA was used alone or IBA and IAA were used in combinations. This suggests that BA is necessary only in shoot production and it should be absent or used at low concentrations during rooting. By using BA, about 750 plantlets can be obtained from a single node shoot of trifoliate orange within 6 months. According to Roy et al. (1990), in vitro regenerated shoots of jackfruit (*Artocarpus heterophyllus* Lam.) produced 5 to 6 new shoots within 30 days when re-inoculated in MS medium supplemented with 1 mg · liter^{-1} BAP and 0.5 mg · liter^{-1} kinetin and this shoot development capacity was retained up to six subcultures. Starrantino and Caruso (1987) propagated some citrus rootstocks in vitro and have reported that after numerous subcultures, the regenerative power is not lost. However, in the case of trifoliate orange, we recommend that sub-culturing should be done only up to the fourth generation because shoot and root development was observed to decline after this point.

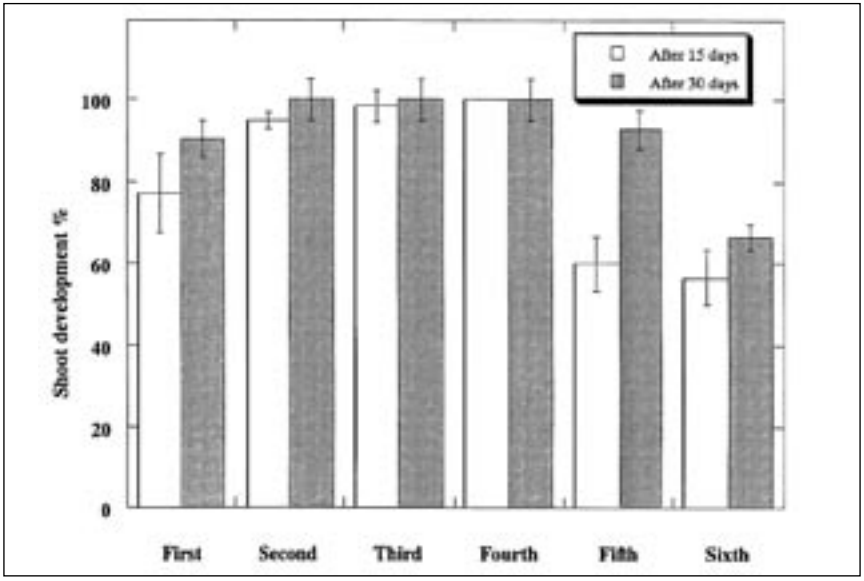

Figure 1. Shoot development percentage of trifoliate orange cultured in vitro from first to sixth generation. Shoot development percentage was evaluated 15 and 30 days after shoot transplanting into the tubes. Bars represent SE.

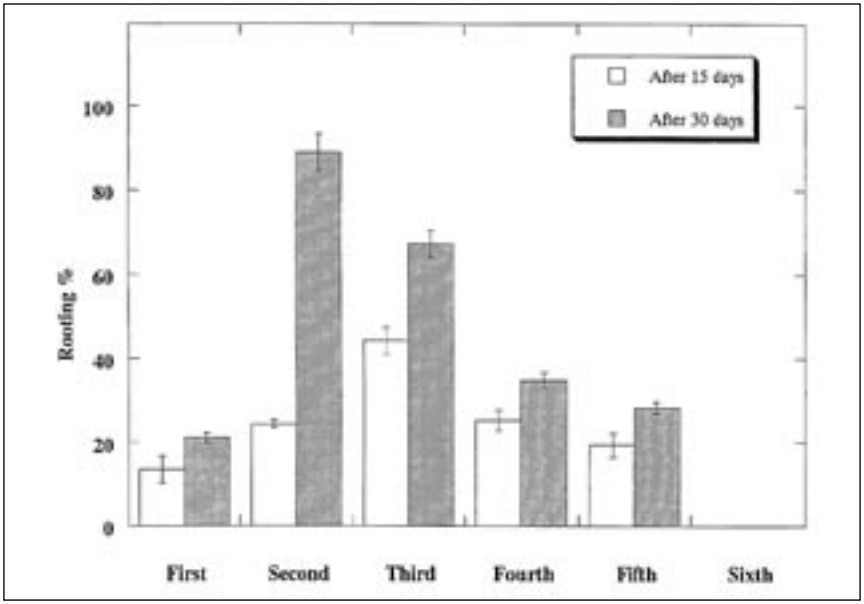

Figure 2. Rooting percentage of trifoliate orange cultured in vitro from first to sixth generation. Rooting was evaluated 15 and 30 days after shoot transplanting into the tubes. Bars represent SE.

Table 2. Effect of IAA, IBA, NAA, and BA combinations on root and shoot development in trifoliate orange cultured in vitro. Root and shoot development was measured 30 days after shoot transplanting into the tubes. Bars represent ± SE.

Treatment	Rooting (%)	Root No.	Root length (cm)	Shoot No.	Shoot length (cm)
1) Control	0	0	0	0	0
2) IAA	100 ± 0	8.7 ± 0.7	1.5 ± 0.08	1.0 ± 0	1.1 ± 0.03
3) NAA	100 ± 0	7.61 ± 0.4	1.18 ± 0.1	1.0 ± 0	1.23 ± 0.23
4) IBA	50 ± 5.7	1.0 ± 0	1.3 ± 0.07	1.0 ± 0	1.0 ± 0.15
5) IBA + IAA	100 ± 0	8.9 ± 0.45	1.53 ± 0.14	1.0 ± 0	1.5 ± 0.27
6) IBA + NAA	100 ± 0	11.1 ± 0.67	0.95 ± 0.17	0	0
7) IAA + NAA	100 ± 0	9.73 ± 0.5	0.87 ± 0.1	0	0
8) IBA + IAA + NAA	100 ± 0	8.4 ± 0.2	0.64 ± 0.07	0	0
9) IAA + BA	0	0	0	2.3 ± 0.17	1.38 ± 0.19
10) NAA + BA	0	0	0	2.2 ± 0.13	1.32 ± 0.2
11) IBA + BA	0	0	0	2.61 ± 0.4	0.79 ± 0.1
12) IBA + IAA + NAA + BA	0	0	0	1.73 ± 0.2	1.4 ± 0.24
13) IBA + IAA + BA	0	0	0	3.2 ± 0.4	1.3 ± 0.15
14) IBA + NAA + BA	0	0	0	2.1 ± 0.1	1.19 ± 0.13
15) NAA + IAA + BA	0	0	0	1.9 ± 0.19	1.6 ± 0.2
16) BA	0	0	0	3.56 ± 0.6	1.34 ± 0.24

The concentration for each regulator was 1 mg liter^{-1} in all combinations.

Hartmann et al. (1997) reported that success in micropropagation of woody plants is to a large extent a function of the juvenility status of the source plant and the micropropagation sequence of subculturing has resulted in rejuvenation that ultimately leads to increased competence for rooting. Previously we reported that stem cuttings collected from mature trifoliate orange trees failed to form callus and to initiate rooting. Stem cuttings from 1-, 2-, 3-, 4- and 5-year-old trifoliate orange trees formed roots to varying degrees, where as those from 15- and 25-year-old trees failed to root (Bhusal et al., 2001, 2002b). The drastic decline in the rooting capacity of trifoliate orange stem cuttings with increasing tree age, and the gradual decrease in rooting after the third generation in trifoliate orange shoots cultured in vitro may be related. This is an interesting subject for further investigation.

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