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**SHORT COMMUNICATION**

# MICROPROPAGATION OF TRIFOLIATE ORANGE (*PONCIRUS TRIFOLIATA*)

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**An attempt was made to develop an *in vitro* regeneration protocol for trifoliate orange (*Poncirus trifoliata*), the important *Phytophthora* collar rot tolerant rootstock genotype of citrus. Among different plant growth regulators used, 1 mg/l BAP, 0.5 mg/l NAA, and 40 mg/l adenine sulphate significantly improved culture response including percentage forming shoot buds, days required for shoot initiation, shoot length and number of resultant shoots per explant. Rooting was achieved on half-strength MS medium supplemented with NAA and IBA either in combination or individually at different concentrations. Medium containing 0.5 mg/l IBA + 0.5 mg/l NAA gave the maximum root initiation response. However, the higher number of roots per shoot and highest root length was achieved when half-strength MS medium was fortified with 0.5 mg/l NAA and 0.5 mg/l IBA respectively. The hardening medium consisted of cocopeat and soilrite (2:1) in which the survival rate of hardened plants was found to be 82.2% after one month.**

***Key words:*** **Micropropagation, protocol, citrus**

Among the different biotic as well as abiotic stresses, citrus cultivation is being devastated by mainly gummosis, i.e. *Phytophthora* collar rot as biotic stress and soil salinity as abiotic stress. Trifoliate orange (*Poncirus trifoliata*) is recognized as *Phytophthora* collar rot tolerant citrus rootstock genotype (Singh 1999). *In vitro* multiplication is a techno-economically viable and eco-friendly approach, which provides an opportunity to screen the variants for different parameters including abiotic and biotic stresses (Thorpe and Harry 1997). However, a standardized protocol for rapid and high regeneration capacity is a prerequisite. Therefore, the present study was undertaken to develop a protocol for *in vitro* regeneration of trifoliate orange.

Fresh seeds of trifoliate orange extracted from just mature fruits were surface sterilized by agitating in 0.1% $HgCl_2$ followed by three rinses in sterilized double-distilled water. These sterilized seeds were inoculated on MS (Murashige and Skoog 1962) basal medium containing 3% sucrose and incubated at $26\pm1^0$C under 16/8 h photoperiod (37 µmol $m^{-2}$ $s^{-1}$). The explants (shoot tip and nodal segment) of 1.0 to 1.5 cm size were excised from *in vitro* grown seedlings and were cultured on MS medium supplemented with different plant growth regulators (NAA, BAP) and adenine sulphate (AS) either alone and/or combinations. All the media were jelled with Difco-bactoagar (0.8%) and pH was adjusted to 5.8 prior to autoclaving ($121^0$C for 20 min at 1.06 kg $cm^{-2}$).

Micro-shoots having 2 cm height or four-leaf stage were cultured for rooting on half-strength MS basal medium supplemented with NAA and IBA either

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individually or in combinations. The well-rooted plantlets were hardened *in vitro* in jam bottles having cocopeat and soilrite (2:1) for one month and then transferred to mist chamber for *ex vitro* establishment. The data was analyzed in completely randomized block design (CRBD) as described by Panse and Sukhatme (1967).

The nodal segment explant and shoot tips cultured on MS basal medium devoid of plant growth substances did not show any response. Hence, the MS medium was supplemented with different concentrations of BAP in combination with NAA and also AS. The explants showed considerable variations in their shoot initiation response to various growth regulators used. The shoot initiation response increased when BAP was supplemented with NAA, and it further increased with the addition of adenine sulphate (AS) (Table 1). Shoot initiation response in shoot tips was slightly higher (87.9%) than in nodal segment (85.9%). The shoot initiation was observed earlier in shoot tip (9 days) than nodal segments (10 days). The number of shoots per explant was higher in nodal segment (4.4 shoots/ explant) than the shoot tip explant (3.6 shoots/ explant). The MS medium 1 mg/l BAP + 0.5 mg/l NAA + 40 mg/l AS proved best for the shoot initiation, days required for shoot initiation, number of shoots per explant and shoot length (Table 1).

The selection of nodal segment for multiple shoots proliferation has been earlier emphasized in *C. sinensis* (Mohanty *et al*. 1998) and in *C. aurantifolia* (Al-Khayri and Al-Bahrany 2001). About 13 to 19 days were required for shoot initiation from nodal segment explants and average 7 shoots per explant were recorded in medium supplemented with cytokinin and auxin in case of *C. sinensis* (Mohanty *et al*. 1998). Highest 8.0 shoots per explant were recorded in *C. aurantifolia* from nodal segment on MS + 1.0 mg/l BAP + 0.5 mg/l Kin (Al-Khayri and Al-Bahrany 2001). A significant effect of adenine sulphate (40 mg/l) with the BAP and NAA for the enhanced shoot proliferation in different citrus species has also been reported (Starrantino and Caruso 1988).

Microshoots (2-3 cm length) developed from both the explant sources were excised and cultured for *in vitro* rooting on ½ strength MS medium supplemented with NAA, and also in combination with IBA (Table 2). The microshoots cultured on MS medium alone did not respond. The auxins evoked different responses when used either alone or in combination (Table 2). The highest root initiation response (93.7%) was observed on ½ strength MS medium containing 0.5 mg/l NAA + 0.5 mg/l IBA. *In vitro* roots were initiated earlier (7.9 days) in the presence of 0.5 mg/l NAA. Longer roots (3.4 cm)

**Table 1:** Effect of phyto-hormones on *in vitro* shooting response of *Poncirus trifoliata*

| Treatment (mg/l) | Shoot initiation response (%) | | No. days req. for shoot initiation | | No. shoots per explant | | Shoot length (cm) | |
|---|---|---|---|---|---|---|---|---|
| | Shoot tip | Node | Shoot tip | Node | Shoot tip | Node | Shoot tip | Node |
| 1.0 BAP | 59.6 | 52.4 | 10.3 ± 0.28 | 10.4 ± 0.33 | 1.2 ± 0.12 | 1.8 ± 0.16 | 0.75 ± 0.21 | 0.61 ± 0.21 |
| 1.5 BAP | 57.7 | 56.3 | 10.2 ± 0.34 | 10.4 ± 0.32 | 1.6 ± 0.16 | 1.4 ± 0.15 | 0.63 ± 0.20 | 0.51 ± 0.20 |
| 1.0 BAP + 0.5 NAA | 81.3 | 74.7 | 9.4 ± 0.31 | 10.3 ± 0.35 | 2.1 ± 0.14 | 2.7 ± 0.19 | 2.52 ± 0.19 | 1.93 ± 0.19 |
| 1.5 BAP + 0.5 NAA | 73.3 | 64.2 | 10.2 ± 0.33 | 10.5 ± 0.31 | 2.2 ± 0.18 | 2.4 ± 0.18 | 2.91 ± 0.18 | 2.65 ± 0.19 |
| 1.0 BAP + 0.5 NAA + AS 20 | 77.7 | 66.2 | 9.2 ± 0.29 | 10.5 ± 0.29 | 3.2 ± 0.14 | 3.9 ± 0.17 | 2.47 ± 0.19 | 2.29 ± 0.18 |
| 1.0 BAP + 0.5 NAA + AS 40 | 87.9 | 85.9 | 8.9 ± 0.28 | 9.7 ± 0.29 | 3.6 ± 0.15 | 4.4 ± 0.13 | 2.33 ± 0.17 | 2.39 ± 0.20 |
| 1.0 BAP + 0.5 NAA + AS 60 | 81.2 | 81.4 | 9.4 ± 0.25 | 10.4 ± 0.28 | 3.4 ± 0.15 | 4.2 ± 0.14 | 2.69 ± 0.16 | 2.48 ± 0.27 |
| CD at 5% | 5.7 | 9.6 | 0.13 | 0.12 | 0.14 | 0.11 | 0.14 | 0.12 |

(i) Sixty to seventy culture tubes in duplex were inoculated with each explant in each treatment and mean values are given in the table, (ii) Values given after ± sign are the standard error calculated by the three replicates where each replication consisted of 10 randomly selected ten test tubes.

**Table 2.** Effect of different phyto-hormones on *in vitro* rooting response of *Poncirus trifoliata*

| Treatment | Parameters | | | |
|---|---|---|---|---|
| (mg/l) | Rooting response (%) | No. of days | No. of roots | Root length (cm) |
| 0.5 NAA | 90.2 | 7.9 ± 0.26 | 5.2 ± 0.19 | 2.3 ± 0.09 |
| 0.5 IBA | 90.6 | 8.3 ± 0.29 | 4.2 ± 0.13 | 3.4 ± 0.06 |
| 0.5 NAA + 0.5 IBA | 93.7 | 7.8 ± 0.23 | 5.3 ± 0.19 | 3.2 ± 0.10 |
| 1.0 NAA + 0.5 IBA | 93.2 | 8.3 ± 0.20 | 4.7 ± 0.20 | 2.6 ± 0.12 |
| 0.5 NAA + 1.0 IBA | 92.4 | 8.3 ± 0.19 | 4.8 ± 0.23 | 2.9 ± 0.14 |
| CD at 5% | 0.14 | 0.18 | 0.12 | 0.11 |

(i) Sixty to seventy culture tubes in duplex were inoculated with each explant in each treatment and mean values are given in the table, (ii) Values given after ± sign are the standard error calculated by the three replicates where each replication consisted of 10 randomly selected ten test tubes.

were developed at 0.5 mg/l IBA where as 0.5 mg/l NAA induced maximum number of roots (5.2) per shoot. Both the percentage and number of roots/shoot increased markedly when NAA was used in rooting medium and the addition of IBA (2.6 μM) enhanced the rooting to 86% in the presence of NAA (2.7 μM) in Troyer citrange (Moreira-Dias *et al*. 2000). The best rooting response (63.33%) has earlier been observed on ½ strength MS supplemented with 0.5 mg/l IBA + 0.5 mg/l NAA, whereas higher concentration of IBA, i.e. 1.0 mg/l IBA + 0.5 mg/l NAA proved beneficial for maximum number of roots (4.16 roots/shoot) followed by 0.5 mg/l IBA + 0.5 mg/l NAA to give 3.91 roots/shoot (Kumar *et al*. 2001). The root length was maximum (3.43 cm) in the presence of 1.0 mg/l NAA followed by 2.99 cm with 1.0 mg/l IBA + 0.5 mg/l NAA in Kinnow mandarin. A minimum of 14.40 days were required for root initiation with 4.04 roots/shoot and 2.0 cm root length on 2.0 mg/l IBA followed by 18.40 days required for root initiation with 3.20 roots/shoot and 1.46 cm root length on 2.0 mg/l NAA in *C. aurantifolia* (Rana and Singh, 2002). Root initiation was observed in 3 weeks after transfer to rooting medium and about 5 to 6 roots/shoot and 4-5 cm root length was achieved on rooting medium supplemented with 1.0 mg/l IAA (Al-Khayri and Al-Bahrany 2001).

The success in hardening of *in vitro* raised plantlets substantiates the viability of the protocol standardization. The survival rate of well-rooted *in vitro* hardened plantlets was 82.1% *in vitro*. The subsequent transfer of the plantlets in the mist chamber reduced the survival rate to 62.7%. Kumar *et al.* (2001) reported the 66% and 67% plantlet survival during hardening of Mosambi and Jaffa sweet orange. The reduced survival in hardening process during the acclimatization is attributed to water stress (Brainerd and Funchigami 1981) and the transition of plantlets from heterotrophic to an autotrophic condition that leads to physiological changes (Wetzestein and Sommer 1962).

In this study the protocol for micropropagation of trifoliate orange (*P. trifoliata*) was standardized. The medium MS + 1 mg/l BAP + 0.5 mg/l NAA + 40 mg/l AS proved the best growth regulator combination for the *in vitro* shoot initiation response. The half- strength MS + 0.5 mg/l IBA + 0.5 mg/l NAA combination performed well for *in vitro* rooting. The observation on hardening medium which consisted of cocopeat and soilrite (2:1), were also encouraging with 82.2% survival of *in vitro* raised plantlets.

## REFERENCES

Al-Khayri, J.M. and Al-Bahrany, A.M. (2001). *In vitro* micropropagation of *Citrus aurantifolia* (lime). *Curr. Sci*. **81:** 1242-1246.

Brainerd, K.E. and Funchigami, L.H. (1981). Acclimatization of aseptically culture apple plants to low humidity. *J. Amer. Soc. Hort. Sci*. **106:** 515-518.

Kumar, K., Dhatt, A.S. and Gill, M.I.S. (2001). *In vitro* plant regeneration in Kinnow mandarin (*C. nobilis* Lour. x *C. deliciosa* Tenora). *Indian J. Hort*. **58:** 299-302.

Mohanty, S., Deka, P.C. and Bhattacharya, S. (1998). Micropropagation of *Citrus sinensis* cultivar Musambi. *Indian J. Agric Sci*. **68:** 114-116.

Moreira-Dias, J.M., Molina, R.V., Bordon, Y., Guardiola J.L. and Garcia, L.A. (2000). Direct and indirect shoot organogenic pathways in epicotyl cuttings of Troyer citrange differ in hormone requirements and in their response to light. *Ann. Bot*. **85:** 103-110.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant*. **15:** 473-497.

Panse, V.G. and Sukhatme, P.T. (1967). Statistical Methods for Agricultural Workers. Indian Council of Agricultural Research, New Delhi.

Rana, J.S. and Singh, R. (2002). *In vitro* clonal propagation of Kagzi lime (*Citrus aurantifolia* Swingle) through shoot tips. *Prog. Hort*. **34:** 27-34.

Singh, S.P. (1999). Citrus industry in Asia. *Souv. Int. Sym. Citriculture*, N.R.C.C., Nagpur, India (Nov. 23-27), 46-67.

Starrantino, A. and Caruso, A. (1988). *In vitro* culture for citrus micropropagation. *Acta Hort*. **227:** 444-446.

Thorpe, T.D. and Harry, I.S. (1997). Application of tissue culture to horticulture (Review). In: Altman, A. and Ziv, M., (eds) Horticultural Biotechnology. *In vitro* culture and breeding. *Acta Hort*. **447:** 39-49.

Wetzestein, H.Y. and Sommer, H.E. (1962). Leaf anatomy of tissue cultured *Liquidambar stracufiura* during acclimatization. *Amer. J. Bot*. **69:** 1579-1586.