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Note

In vitro regeneration through direct shoot organogenesis in Honey Orange (*Citrus tangerina*)

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Abstract Rapid propagation of Honey orange (*Citrus tangerina*) was achieved by induction of shoots from epicotyl and cotyledonary node explants and rooting of cotyledonary node derived shoots. Significant explant differences were observed in the induction of direct shoots. Cotyledonary node explant is the most efficient in regeneration frequency followed by epicotyl explant. Cotyledonary node explants cultured on Murashige and Skoog (MS) medium supplemented with 8.88 μM *N*6-benzyladenine (BA) and 0.54 μM α-naphthaleneacetic acid (NAA) developed more than five shoots per explant. The isolated shoots transferred onto the MS medium supplemented with 5.4 μM NAA rooted 100% within 30 days.

Key words: Direct organogenesis, honey orange, seedling explants.

In Myanmar, Honey tangerine (*Citrus tangerina*) is well known as Honey orange. It is one of the most commercially important cultivars due to its adaptability to climatic conditions of Myanmar and high yield preferred by growers and its quality fruits preferred by consumers. Propagation by conventional budding and grafting techniques could not provide enough planting materials to keep pace with the demand. In vitro propagation can provide opportunities for rapid mass propagation of a new variety within short time. Citrus in vitro regeneration in several species has been reported using shoot tip, stem and epicotyl segments, roots, leaf sections and reproductive organs as explants (Chaturvedi et al. 2001; Costa et al. 2004). Responses to conditions of in vitro culture may vary depending upon genotype, explant type and orientation, composition of the culture medium, and conditions of incubation (Costa et al. 2004; Perez-Tornero et al. 2010). Shoot induction and rooting of various citrus explants have been found to be strongly affected by the concentration of plant growth regulators (PGRs) in the culture medium. Both shoot and root initiations in numerous citrus species were positively influenced by PGRs, *N*6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA) (Costa et al. 2004; Singh and Rajam 2009). But PGRs requirement for optimal shoot regeneration varies and is considered to be genotype-specific (Barlass and Skene 1986). However, up to our knowledge, there is no report on regeneration of Honey orange. Therefore in this study, a regeneration protocol for Honey orange was studied aiming to achieve rapid propagation within a short time.

Shoot tip (0.5–1.0 cm), epicotyls (0.5–1.0 cm) and cotyledonary node (0.5–1.0 cm) explants derived from two week old seedlings of Honey orange were used as the source of explants and cultured on MS (Murashige and Skoog 1962) medium (Figure 1). In the present study, MS medium was modified with different concentrations of

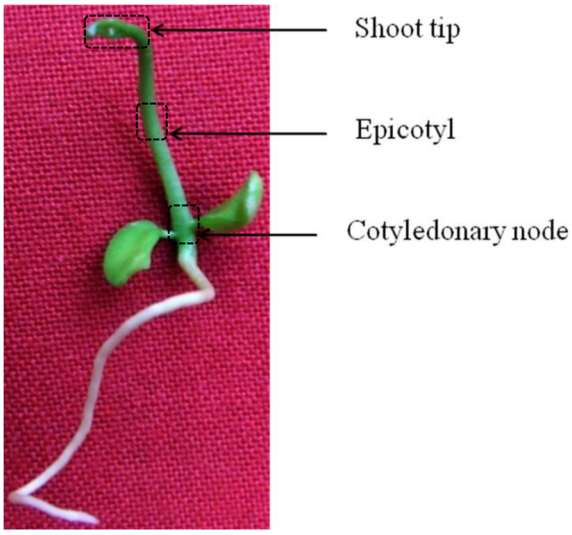

Figure 1. In vitro seedling of Honey orange (2–3 cm).

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Table 1. Effects of different combinations and concentrations of NAA and BA on the shoot regeneration frequency (%) of different kinds of explants in Honey orange.

PGRs (μM)*		Shoot formation (%)			No. of shoots per explant	
BA	NAA	Epicotyl	Nodal	Shoot tip	Epicotyl	Nodal
4.44	0.54	50.0± 1.83 a	76.0± 1.58 a	0	1.4±0.40 a	3.0±0.45 b
8.88	0.54	42.0± 1.22 b	71.0± 0.91 a	0	1.0±0.00 a	5.6±0.60 a
22.20	0.54	21.0± 0.91 c	39.0± 1.83 b	0	1.6±0.24 a	2.2±0.49 b

Data were recorded 8 weeks after culture period. The values indicate the means and the standard errors. Means in each column followed by the same letter are not statistically different by Duncan's Multiple Range Test (DMRT) at $p=0.05$. * PGRs=Plant Growth Regulators, NAA=Naphthaleneacetic acid, BA=Benzyladenine.

BA (4.44–22.2 μM) in combination with NAA (0.54 μM). All media were supplemented with 3% (w/v) sucrose, and gelled with 0.6% (w/v) agar. The pH of the medium was adjusted to 5.7 before gelled with agar. All the media were sterilized at a pressure of 1.06 kg cm^{-2} (121°C temperature) in an autoclave for 20 min. Test tubes (25 mm×200 mm) and jam bottles (50 mm×125 mm) were used as culture vessels. Test tubes and jam bottles were dispensed with 20 and 60 ml medium respectively. Aluminium foil was used as closures for test tubes, while polypropylene autoclavable lids were used for jam bottles. All cultures were incubated at 25±2°C with 16 h light (at an irradiance of 30 μmol m^{-2} s^{-1})/8 h dark cycle. All three types of explants were cultured horizontally and at least 60 explants were cultured per treatment. Survival (%) was recorded 4 weeks after culture period. Shoot formation frequency (%), number of shoots and shoot buds per explant were recorded after 8 weeks culture period.

Multiple shoot clumps derived from nodal explants were used for rooting. Shoots of 0.5–1.0 cm in height were detached individually from the shoot clusters and cultured on MS medium supplemented with different levels of NAA (5.4–16.1 μM) for rooting. Root formation and plantlet regeneration were recorded 4 weeks after culture period. At least 30 plantlets were measured per treatment. The experiments were run in a completely randomized design. Treatment means were compared by Duncan's Multiple Range Test at 5% level.

Almost all shoot tip, epicotyl and cotyledonary node explants survived in the basal MS media supplemented with BA and NAA. Within one week, nodal section and epicotyl explants showed swelling at the cutting edges, from which adventitious shoots developed after 2 weeks in culture. Only some adventitious shoots continued to elongate to form shoots. BA and NAA were found to be proper plant growth regulators for the induction of direct shoots from the cotyledonary node and epicotyl explants. Evaluations for response were done after 8 weeks culture period, when shoots higher than 5 mm were counted. No morphogenic response was observed in shoot tip explants although they were still green. Shoot formation frequency was observed in descending order from cotyledonary node (74%) and epicotyl (50%) to shoot tip (0%) explants (Table 1). The morphogenic gradient along the epicotyl axis was observed and cotyledonary node showed higher regeneration ability than middle epicotyl portion and shoot tip. The results are in agreement with those by Burger and Hackett (1986) for Valencia orange (*Citrus sinensis* L. Osb.), by Garcia-Luis et al. (1999) and Moreira-Dias et al. (2001) for Troyer citrange (*C. sinensis* L. Osb.×*Poncirus trifoliata* L. Raf.). The expression of the morphogenic gradient along the epicotyl axis was assumed mainly influenced by composition of the culture medium. Similar results were reported when the culture medium contained both BA and NAA (Burger and Hackett 1986; Costa et al. 2004). Burger and Hackett (1986) reported that epicotyl sections of Valencia orange lose the ability to form adventitious buds as distance from the cotyledonary node increases on the culture medium supplemented with BA and NAA. In the present study, shoot tip explants showed no morphogenic response although all cultured explants were alive till 8 week after culture. No buds formed on the epicotyl sections with or without an apex taken from tissue near the apex (Burger and Hackett 1986). However, in vitro response of shoot tip explants may be dependent on cultivar. Carimi and De Pasquale (1999) reported that shoot tip explants of Rangpur lime, sweet orange and trifoliate orange did not respond well in culture, whereas Carrizo citrange shoot tips proliferated vigorously. Further studies are necessary to investigate limiting factors in shoot proliferation of shoot tip explants in this variety. Conflicting reports that expressed a higher organogenic response as the distance of the explants from the cotyledonary node increased were also reported when the culture medium was supplemented with BA only for *C. mitis* by Sim et al. (1989), for *C. grandis* by Goh et al. (1995) and for 'Cravo' rangpur lime (*Citrus limonia*), 'Foster' grapefruit (*C. paradisi*) and 'Pera' sweet orange (*C. sinensis*) Costa et al. (2004). Another factor influencing the gradient was assumed as concentration of BA and NAA in the culture medium. Higher concentrations of BA (4.44–22.2 μM) than NAA (0.54 μM) were used in this study. The gradient was expressed when there were higher concentrations of BA than NAA and not expressed when there were equal concentrations of BA and NAA in the medium (Burger and Hackett 1986). In cotyledonary node explant, PGR combination, 8.88 μM BA and 0.54 μM NAA

Figure 2. Direct multiple shoots of 8 weeks after culture (A) and well rooted plantlet (2–3 cm) with roots of 12 weeks after culture (B) developed from nodal explant. Arrows show regenerated shoots or shoot buds (A) and roots (B).

was superior and resulted in significantly more shoot number (5.6 shoots per explant) (Table 1, Figure 2A). Shoot induction was found directly proportionate to the increase in level of BA up to a point (8.88 μM) and further increment (22.2 μM) showed decline in shoot induction. The results seem to follow those of Begum et al. (2004) in which regeneration per nodal explant was the best on the medium containing 4.44 μM BA and further increase in BA reduced number of shoots per explant in all 3 varieties of pummelo. Similar results were reported by Ali and Mirza (2006) in which 13.2 μM BA was the best for shoot regeneration from stem explant of rough lemon (83%) and further increase in BA reduced shoot regeneration efficiency.

Epicotyl explants gave maximum number of shoots per explant (1.6) in this study on the medium containing 22.2 μM BA and 0.54 μM NAA (Table 1, Figure 3A, B). But it was not significantly different from the one on the medium with 4.44 μM BA and 0.54 μM NAA which gave maximum shoot formation frequency (50%) in epicotyl explants. It might be assumed that 4.44 μM BA and 0.54 μM NAA was optimum concentration for epicotyl explants in Honey orange. In addition, Usman et al. (2005) reported that 4.44 μM BA was the best for shoot induction from epicotyl explants of Natal, Valencia and Hamlin, with averages of 1.59, 1.76 and 2.43 shoots per explant, respectively.

In Citrus, small shoot rooted very poorly. Therefore, only shoots with a minimal length of 0.5 cm were transferred to rooting medium. It was observed that frequency of root formation was 100% on the medium supplemented with 5.4 μM NAA from the shoots cultured on the media containing 4.44 μM BA (Figure 2B). Lower root formation frequencies of 54% and 47% were observed on media with 10.8 and 16.1 μM NAA respectively. Carimi and De Pasquale (1999) reported similar findings in sweet orange. Beneficial effects of

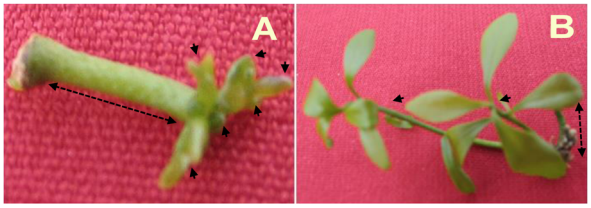

Figure 3. Direct shoot-buds induced from an epicotyl explant shown by arrow (A) and well developed multiple shoots from the explant shown by arrow (B) after 8 weeks culture. The length of the original explant was 5–10 mm and shown by dotted arrows.

NAA in rooting of various citrus cultivars were reported by Ali and Mirza (2006), Kitto and Young (1981). So, it might be assumed that 5.4 μM NAA was the optimum concentration for root induction of the shoots derived from nodal explant. These results suggested that 5.4 μM NAA can be used for rooting of the shoots derived from nodal explants of Honey orange.

Up to our knowledge, this is the first report on regeneration of Honey tangerine or Honey orange. Cotyledonary node explant was found to be superior to epicotyl and shoot tip explants. MS medium supplemented with 8.88 μM BA and 0.54 μM NAA was efficient for shoot induction. One hundred percent root formation frequency was recorded on the medium supplemented with 5.4 μM NAA. In this study, plantlets were regenerated from two week old seedlings and this system might be suitable to achieve high propagation efficiency for Honey orange.

References

Ali S, Mirza BU (2006) Micropropagation of rough lemon (*Citrus jambhiri* Lush.): Effect of explants type and hormone concentration. *Acta Botany Croatica* 65: 137–146

Barlass M, Skene KGM (1982) In vitro plantlet formation from Citrus species and hybrids. *Sci Hortic* (Amsterdam) 17: 333–341

Begum F, Amin MN, Islam S, Azad MAK (2004) A comparative study of axillary shoot proliferation from nodal explants of three varieties of pummelo (*Citrus grandis* Osb. L.). *Biotechnology* 3: 56–62

Burger DW, Hackett WP (1986) Gradients of adventitious bud formation on excised epicotyl and root sections of citrus. *Plant Sci* 43: 229–232

Carimi F, De Pasquale F (1999) Micropropagation of Citrus. In: Morhan JS, Ishii K (eds) *Micropropagation of woody trees and fruits*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 589–604

Chaturvedi HC, Singh SK, Sharma AK, Agnihotri S (2001) Citrus tissue culture employing vegetative explants. *Indian J Exp Biol* 39: 1080–1095

Costa MGC, Alves VS, Lani ERG, Mosquima PR, Carvalho CR, Otoni WC (2004) Morphogenic gradients of adventitious bud and shoot regeneration in epicotyl explants of Citrus. *Sci Hortic* (Amsterdam) 100: 63–74

Garcia-Luis A, Bordon Y, Moreira-Dias JM, Molina RV, Guardiola JL (1999) Explant orientation and polarity determine the morphogenic response of epicotyl segments of Troyer citrange.

Ann Bot (Lond) 84: 715–723

Goh CJ, Sim GE, Morales CL, Loh CS (1995) Plantlet regeneration through different morphogenic pathways in pommelo tissue culture. *Plant Cell Tiss Org Cult* 43: 301–303

Kitto SL, Young MJ (1981) Micropropagaion of Carrizo Citrange. *Horticultural Science* 16: 305–306

Moreira-Dias JM, Molina RV, Guardiola JL, Garcia-Luis A (2001) Daylength and photon flux density influence the growth regulator effects on morphogenesis in epicotyl segments of Troyer citrange. *Sci Horti* 87: 275–290

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Perez-Tornero O, Tallon CI, Porras I (2010) An efficient protocol for micropropagation of lemon (*Citrus limon*) from mature nodal segments. *Plant Cell Tiss Org Cult* 100: 263–271

Sim GE, Goh CJ, Loh CS (1989) Micropropagation of *Citrus mitis* Blanco-multiple bud formation from shoot and root explants in the presence of 6-benzylaminopurine. *Plant Sci* 59: 203–210

Singh S, Rajam MV (2009) Citrus biotechnology: Achievements, limitations and future directions. *Physiology and Molecular Biology of Plants* 15: 3–22

Usman M, Sana M, Fatima B (2005) Multiple shoot induction from nodal explants of citrus cultivars. *J Cent Eur Agric* 6: 435–442