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In Vitro Regeneration and Somatic Embryogenesis in (*Citrus aurantifolia* and *Citrus sinensis*)

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ABSTRACT

Most of the plant regeneration processes in citrus, through tissue culture, involve somatic embryogenesis. The optimization of these processes is important for the development of *in vitro* plant improvement. Nodal segments and leaf discs of sweet orange (*Citrus sinensis* L.) cv. Musambi and Lime (*Citrus aurantifolia*) cv. Kaghzi Nimbu were used to obtain aseptically raised plantlets of Lime (*Citrus aurantifolia*) cv. Kaghzi Nimbu and sweet orange (*Citrus sinensis* L.) cv. Musambi on MS media supplemented with 2,4-D (2 mg L^{-1}) and coconut milk (20%). Callus induction was highest when shoot segment of lemon were cultured on MS media supplemented with 2, 4-D and coconut milk. Embryo proliferation was highest when they were cultured on MS media supplemented with 1.5 mg L^{-1} of Kinetin. Shoot induction was highest when embryos were cultured on MS media supplemented with 2.0 mg L^{-1} of Benzyl amino purine (BAP) in both species. Highest rooting percentage in Musambi was obtained at concentration of 1.5 mg L^{-1} NAA.

Key Words: Somatic embryogenesis; Musambi; Kaghzi Nimbu; Callus induction; Citrus

INTRODUCTION

Sweet orange and lemon are highly popular and commercially cultivated for its processing quality, fresh consumption and aromatic flavor. Despite its excessive cultivation, citrus plantation still has some problems such as slow growth and long juvenility, insects, pests, diseases, alternate bearing, pre and post harvest losses, large number of seeds per fruit, short season of supply and short storage life etc. Traditional genetic plant improvement offers limitation for the production of new varieties of scion and root stocks, and the new varieties produced so far were originated form natural selection and mutation. The first barrier met by researcher was related to the complex citrus biology, which has high nucellar polyembryony, high heterozygosis, auto incompatibility and a long juvenile period (Grosser & Gmitter Jr., 1990).

Advances in biotechnology have generated new opportunities for citrus genetic improvement. *In vitro* propagation has therefore been a great potential tool to overcome problems related with the field culture for such species (Kitto & Young, 1981; Hidaka & Omara, 1989). Techniques like *in vitro* culture made it easy to improve citrus against different a biotic stresses, low yield and conserve important citrus genotypes though exploiting soma clonal variations (Chandler *et al*., 1996), somatic cell hybridization, (Kobayashi, *et al.*, 1992; Deng *et al.*, 2000), transformation of high yielding cultivars (Koltunow *et al.*, 2002) disease free plants (Greno *et al.*, 1988). But all these highly sophisticated technique requires the presence of highly responsive regeneration protocol.

This study was conducted with the aim to explore regenerative ability, appropriate media and explants of sweet orange (*Citrus sinensis* L.) cv. Musambi and Lime (*Citrus aurantifolia*) cv. Kaghzi Nimbu

MATERIALS AND METHODS

The present study was conducted in the Plant Tissue Culture, Institute of Horticultural Sciences, University of Agriculture, Faisalabad–Pakistan.

Plant material. Fruits of Sweet orange cv. Musambi (*C. sinensis* L Osbeck) and Kaghzi Nimbu [*C.aurantifolia* (Christem) Swingle] were collected from Post-graduate Agriculture Research Station (PARS), University of Agriculture, Faisalabad–Pakistan.

Sterilization of seeds. Seeds of citrus species were sterilized by dipping in 95% ethanol for 1 minute and then rinsed 4 times with sterilize double distilled water. Sterilized seeds were peeled and cultured on simple MS media (Murashige & Skoog, 1969) aseptically.

Cultural conditions. After inoculation cultured seeds were placed under dark at 25± 20°C till two weeks to get the etiolated seedlings.

Growth Hormones Concentrations

Callus induction median (CIM). MS media with 2 mg L^{-1} of 2,4-D + 20% coconut milk (under dark).

Embryo induction media (EIM). MS media supplemented Kinetin (0.5, 0.1, 1.5 and 2.0 mg L^{-1}) (EIM_1) NAA (0.1, 0.1, 0.1 and 0.1 mg L^{-1}) (EIM_2).

Shoot induction media (SIM). MS media supplemented with BAP (0.25, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}).

Root induction media (RIM). MS media with different concentrations of NAA (1.0, 2.0 and 1.5 mg L^{-1}).

Explants. The nodal segments (1-1.5 cm), cotyledonary leaves and leaf discs of size $1cm^2$.

RESULTS AND DISCUSSION

Effect of explant on callus induction in *Citrus sinensis* and *Citrus aurantifolia.* The data (Fig. 1) showed that callus induction was highest when shoot segment of lemon were cultured on MS media supplemented with 2, 4-D and

coconut milk. However cotyledons produce highest percentage of callus induction in both species. These results are in agreement with Rani *et al.* (2003), that maximum induction (31.94%) of embryogenic callus was observed on MS medium supplemented with 0.5 and 1.0 mg L^{-1} Kinetin. Gitarani *et al.* (2003), and Haoa *et al.* (2004) got increased callus induction percentage with increasing level of auxins NAA and 2, 4-D in the media

Effect of Kinetin on embryoid formation and shape of embryo in *C. sinensis* and *C. aurantifolia*. The results showed that embryo proliferation was highest when they were cultured on MS media supplemented with 1.5 mg L^{-1} of Kinetin (Fig. 2). In lemon heart shape structure (Embryoids) were obtained with highest success, when they were cultured in MS media supplemented with 0.5 mg L^{-1} of Kinetin. Similar percentages of embryoids were obtained in both species at 2 mg L^{-1} concentration of Kinetin. These results are in accordance with the Mis *et al.* (1995) that MS medium supplemented with 10 mg L^{-1} NAA, 1 mg L^{-1} Kinetin and vitamins were best for induction of somatic embryogenesis in *citrus reticulata*. Gloria *et al.* (2000) observed good embryoids formation due to presence of auxins and good somatic embryo formation occurred on MT medium supplemented with 500 mg L^{-1} of malt extract in culture media.

Effect of Naphthalene acetic acid (NAA) on embryoid formation and shape in *C. sinensis* and *C. aurantifolia*. The results indicated that good friable callus was obtained with highest percentage at 0.1 mg L^{-1} Naphthalene acetic acid (NAA) in both species (Fig. 3). Embryo formation increases with the increase of hormonal level. It was interesting to note that response of both species was almost similar at other 3 concentrations level of NAA. These results got support from Mis *et al.* (1995), who reported that MS medium supplemented with 10 mg L^{-1} NAA, 1 mg L^{-1} Kinetin and vitamins were best for induction of somatic embryogenesis in *Citrus reticulata.* Gloria *et al.* (2000) observed in citrus similar good embryoids formation due to the presence of auxins where as good somatic embryo formation was occurred on MT medium supplemented with 500 mg L^{-1} of malt extract. Tomaz *et al.* (2001), observed the effects of auxins (NAA and BAP) on embryoids formation percentage in citrus and significant results for embryoids formation.

Effect of Benzyl amino purine (BAP) on shoot induction percentage in *Citrus aurantifolia* and *Citrus sinensis*. The highest percentage of shoot induction was obtained when shoot were cultured on MS media supplemented with 2.0 mg L^{-1} of Benzyl amino purine (BAP) in both species i.e. Musambi and Lemon (Fig. 4). Response of shoot induction in both species increases with the increase of hormone level in both species. However shoot induction response was optimum at 1.0 mg L^{-1} of BAP which decreases with the increase of hormone concentration. These results find support from the previous workers like Gloria *et al.* (2000), that plant regeneration was also achieved by adventitious

Fig. 1. Effect of explant on callus induction in sweet orange (*Citrus aurantifolia*) and Khazi Nimbu (*Citrus aurantifolia*)

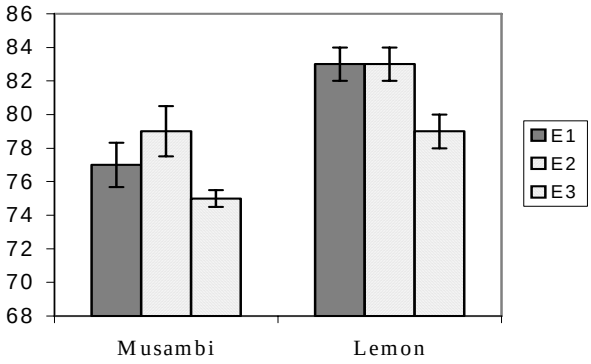


Fig. 2. Effect of kinetin on embryoid formation

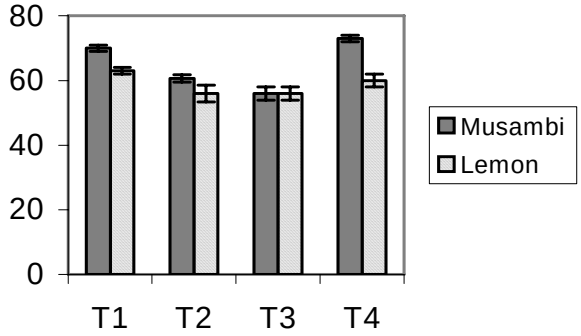
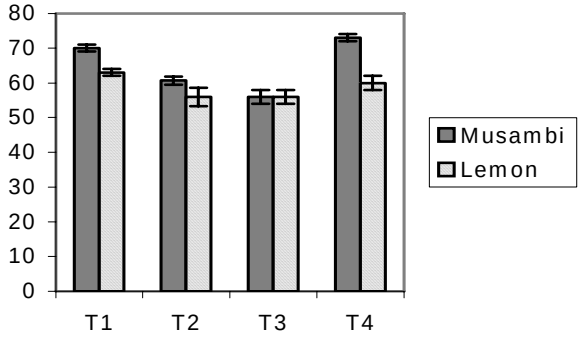


Fig. 3. Effect of naphthalene acetic acid on embryoid formation



shoots obtained through direct organogenesis of not well defined embryos in modified MT medium with addition of malt extract (500 mg L^{-1}), BAP (1.32 M), NAA (1.07 M) and coconut water (10 mg L^{-1}). Paul and Chaudry (2000), Beneditu *et al.* (2000) and Chandra *et al.* (2003) observed good shoot formation percentage in the presence of auxins in culture media.

Effects of Naphthalene acetic acid (NAA) on rooting percentage in *Citrus aurantifolia* and *Citrus sinensis*. The results regarding root formation percentage under different concentrations of NAA, revealed that highest rooting percentage in Musambi was obtained at concentration of 1.5 mg L^{-1} NAA. The rooting percentage increases up to level (T_2) treatment, later it showed decreasing trend (Fig. 5).

Fig. 4. Effect of BAP on shoot induction in citrus

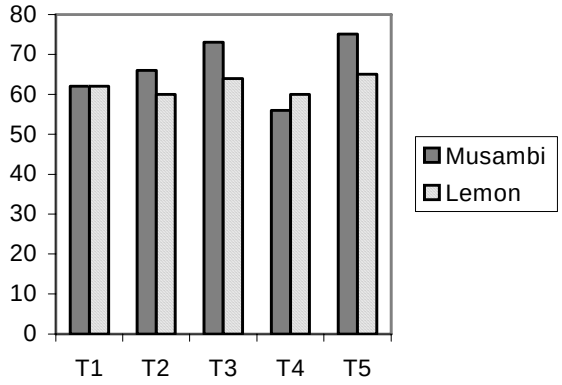
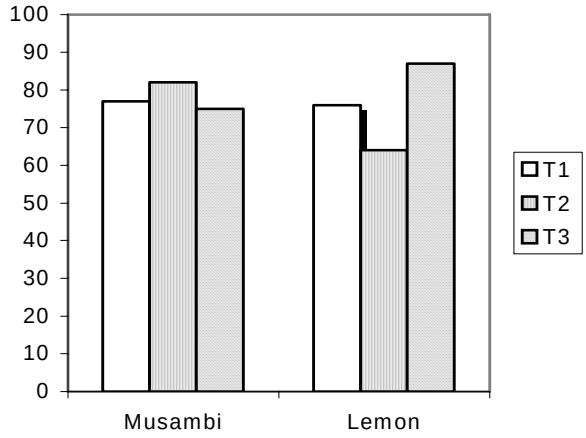


Fig. 5. Effect of NAA on rooting percentage in citrus



Therefore, optimum level of NAA is required for rooting in Musambi. However, in Lemon highest response of rooting was obtained at high level of NAA i.e. 2.0 mg L^{-1}. In Lemon rooting response increases with the increase of hormonal level. These results find support from Obukosia and Waithaka (2000) that the embryos were separated and subcultured in MS media containing coconut milk but without hormones, first developed roots within 4 to 8 months and then shoots within 6 to 9 months of *in vitro* culture. Kim *et al.* (2002) reported that MS media supplemented with 1.5 mg L^{-1} NAA was most effective for root induction in Yooza mandarin; whereas, MS media + 0.1 mg L^{-1} BAP gave best result for shoot induction. Ling *et al.* (2002), and Chandra *et al.* (2003) observed good root formation in the presence of auxins in the culture media.

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