A study Towards an Efficient Protocol for Somatic Embryogenesis in Sri Lankan *Citrus* Species

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Abstract

Higher production of Sri Lankan *Citrus spp.* is needed to reduce the import expenditure. Lack of elite planting material is one of the major barriers for intensive cultivation. Development of an efficient *in vitro* regeneration protocol is essential for elite planting material production and genetic improvement of *Citrus*. This study evaluated the effect of maltose concentration on local *Citrus spp.* for direct somatic embryogenesis followed by plant regeneration. Mature embryos of *Naran* (*Citrus crenatifolia*), *Heennaran*(*Citrus reticulate*) and *Panidodam* (*Citrus sinensis*) were used as explants. In experiment 1, cotyledons of *Naran* were placed in 100mg/l and 200mg/l of maltose with liquid MS to select the effective maltose level. Multiple somatic embryos formed from single *Naran* cotyledons after 5 days of culture. Significantly higher frequency of somatic embryogenesis was observed from 200mg/l maltose level. In experiment 2, *Heennaran*and *panidodam* cotyledons were placed in selected level of 200 mg/l of maltose. Somatic embryos of *Heennaran* cotyledons developed into seedlings of different heights from 0.5 cm to 5 cm after 5 days of cotyledon culture. Percentage of first somatic embryogenesis from total explants was 87%. The secondary somatic embryos were formed in 30% of cotyledons. In *Panidadam*, 78% and 69% of initial and secondary somatic embryos were formed, respectively. A significant difference could not be detected between two species on somatic embryogenesis on 200 mg/l of maltose level in liquid MS culture. Possibility for genetic transformation of *Citrus* was also tested: Cotyledon explants were transformed by *Agrobacterium* bearing 35S: *VlmybA2* for induction of anthocyanin pigments. Putative 35S: *VlmybA2*somatic embryos emerging from transformed cotyledons, produced purple dots after 1 week of culture indicating the potential of transformation. This study indicates the potential of this protocol for efficient somatic embryogenesis for future multiplication and crop improvement programmesthrough genetic transformation of Sri Lankan *Citrus spp.*

Keywords: somatic, embryogenesis, Citrus

Introduction

Higher production of Sri Lankan *Citrus spp.*is an important solution for reducing the expenditure for imported *Citrus* fruits. Lack of elite planting material is one of the major barriers for higher production in Sri Lanka. Genetic improvement of local *Citrus spp* is also important for better fruit quality and disease resistance. Traditional breeding programmes are hampered with high cost and complexity of genetic system of *Citrus*. Therefore, development of an efficient *in vitro* regeneration protocol is very useful for planting material production and genetic improvement of *Citrus* through genetic engineering.

Direct somatic embryogenesis from embryo associated explants is an efficient method for several *Citrus spp* (Button and Kochba, 1977). Somatic embryos are proved to be the excellent material for genetic transformation work in tree species like *Citrus* (Bajaj, 1995). Attempts on development of *Citrus sinensis* plants through direct somatic embryogenesis had been reported in Sri Lanka as well (Geekiyanage *et al.*, 2001). Use of maltose had been reported to induce direct somatic embryogenesis there.

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An attempt was made to check the effect of maltose concentration on the response of different local *Citrus spp* for direct somatic embryogenesis followed by plant regeneration.

The potential of cotyledon explants for genetic transformation was also checked. An anthocyanin pathway gene *VlmybA2*was used as it could be a visual marker with purple anthocyanin induction.

Methodology

Mature embryos of commercially available *Citrus spp.* - *Naran* (*Citrus crenatifolia*), *Heennaran* (*Citrus reticulate*) and *Panidodan* (*Citrus sinensis*) were used as explants. Mature *Citrus* fruits were surface sterilized in running water, followed by 95% ethanol for 10 minutes and 20% Clorox for 20 minutes with several washings in sterile distilled water. Seeds were obtained from cut open fruits. Only excised cotyledons were selected for the culture in modified Murashige and Skoog (MS) liquid medium with maltose. In the first experiment, cotyledons of *Naran* were placed in liquid with both 100mg/l maltose and 200mg/l maltose.

In experiment 2, *Heennaran* and *Panidodam* cotyledons were placed in liquid MS with 200 mg/l maltose only. Observations were made on somatic embryogenesis in the liquid culture.

Cotyledon explants were transformed by *Agrobacterium* bearing pBE 2113/*VlmybA2*. Cotyledons were kept in liquid co cultivation medium of MS and 200 mg/l of maltose for 3 days. After that, explants were washed in distilled water and placed in above medium with 400 mg/l carbenicilin, 100 mg/l cefotaxime and 50 mg/l kanamycin.

Results and Discussion

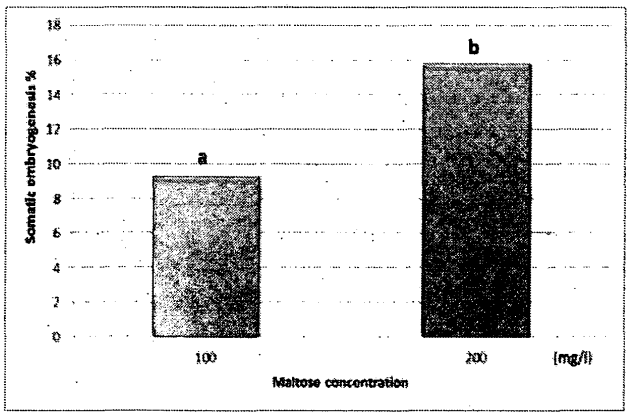
Experiment 1:

Somatic embryos formed in *Naran* cotyledons after 5 days of culture. There were cotyledons with more than 1 somatic embryo. Somatic embryos formed at the cut end of cotyledon and at the opposite edge of the cut end as well. There was a significant difference between frequencies of somatic embryogenesis in 100mg/l maltose level and 200mg/l maltose level (Figure 1.a).

Experiment 2:

Maltose concentration was taken as 200 mg/l in this experiment as it was effective in the previous experiment for *Citrus*.

Somatic embryo formation in *Heennaran* cotyledons was observed. Embryos developed into seedlings of



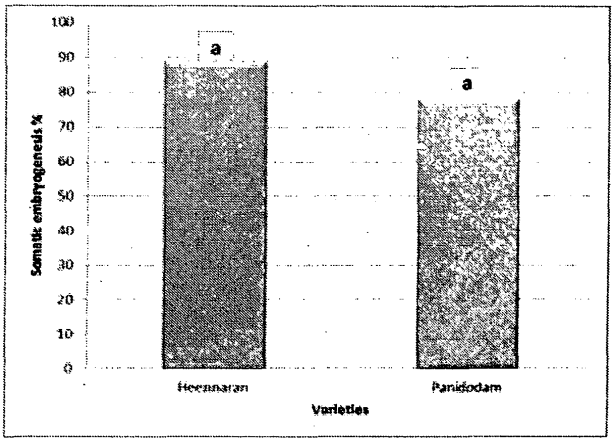


Figure: 1. (a) Effect of maltose concentration on somatic embryogenesis in cotyledons of *Naran* (*C. crenatifolia*). Different letters indicate significant differences at 0.05 probability level. (b) Effect of variety on somatic embryogenesis in cotyledons of *Heennaran* (*C. reticulate*) and *Panidodam* (*C. sinensis*)

different heights ranging from5 cm to 0.5 cm after 5 days of cotyledon culture. Percentage of first somatic embryogenesis from total explants was 87%. The secondary somatic embryos were formed in 30% of cotyledons. In *Panidadam*, 78% and 69% of initial and secondary somatic embryos were formed, respectively. A significant difference could not be detected between two species on somatic embryogenesis (Figure 1.b).

Transformation with pBE 2113/*VlmybA2:*

Purple dots were observed from emerging embryos from cotyledons that are transformed by 35S:*VlmybA2*after 1 week of culture. White somatic embryos formed in control cotyledons.

To confirm the transgenic embryo formation from transformed cotyledons, PCR for *VlmybA 2* is needed.

Conclusion

Both Heennaran (*C. reticulate*) and Panidodam *(C. sinensis)* have the same potential to produce somatic embryos at 200 mg/l maltose level in liquid MS culture.

Tested varieties may be transformed by *Agrobacterium* and *VlmybA2* may induce purple pigmentation in Sri Lankan *Citrus.*

Further work on different concentrations and sugar sources would be useful in achieving optimum somatic embryogenesis percentage in *Citrus* species.

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