In vitro Plant Regeneration of *Cyphomandra betacea* through Nodal Culture

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Abstract

Cyphomandra betacea *(Cav.) is commonly known as tamarillo or tree tomato. This species is mainly used for its edible fruits which have a high nutritional value and contain relatively high amounts of vitamins B6, C, E and provitamin A. The cultivation of tamarillo in Rwanda is facing major challenges caused mainly by viral diseases like tamarillo mosaic virus (TaMV). These pathogens are difficult to control and are transferred by vegetative propagation, often resulting in heavy productivity losses and poor quality fruits. The conventional methods of propagating Tamarillo are both slow and inefficient. This study was conducted to develop an efficient, rapid propagation protocol for C. betacea through in vitro nodal culture. The effect of phytohormones on bud break and subsequent microshoot proliferation from nodal explants was evaluated. The nodal explants were cultured on Murashige and Skoog media supplemented with different concentrations of Benzyl amino purine (BAP), 2- isopentenyl adenine (2iP) and kinetin as well as 100 mg/l myo-inositol 3% sucrose and gelled with 0.3% gelrite. The Tukey test showed that there were highly (p=0.001) significant differences among the different cytokinin levels for microshoot elongation in tamarillo. Although, microshoots developed in all the media evaluated, BAP at 40 µM/l was the most effective in inducing bud break and multiple shoots. This media induced multiple shoots at an average rate of 1.42±0.34 shoots per node and the highest shoot length of 39.25±9.05mm after 35 days in culture. The microshoots were able to root without addition of an exogenous auxin and the plantlets successfully hardened in the greenhouse. The study has developed a very simple one step regeneration protocol which can be adopted to mass propagate Tamarillo. This reproducible protocol opens new prospects for massive propagation of tamarillo and will go a long way in alleviating the shortage of tamarillo disease-free planting materials in Rwanda.*

Key words: *Tissue culture,* ***Cyphomandra betacea,*** *Tamarillo, microshoot*

1. Introduction

The export of tamarillo (*Cyphomandra betacea*) is an important commodity in Rwanda's exotic fruit industry. However, the conventional methods used in propagating *C. betacea* produce a high degree of genetic variability which negatively affects fruit colour, resulting in rejection of fruits on the international market. *C betacea* is also prone to several seed borne diseases in Rwanda which may destroy the seedling in its early stage of development. There is, therefore, urgent need to look for feasible alternatives of propagating the crop. Tissue culture or *in vitro* propagation offers such alternative and large numbers of plants can be regenerated using small explants.

An increasing number of studies describing *in vitro* propagation of tamarillo have been reported. Obando *et al.* (1992) regenerated *Cyphomandra betacea* from axillary buds using MS basal medium and phytohormones Naphylacetic Acid (NAA), BAP and Gibberellic Acid (GA_3) all evaluated at a concentration of 0.2 mg/l. Barghchi (1998) established a protocol for the efficient micropropagation and plant improvement of *Cyphomandra betacea* using explants from axillary and flower buds of mature tamarillo plants cultured on MS medium supplemented with a combination of different growth regulators (BAP 0.1-4.0 mg/l, kinetin 0.25-4.0 mg/l and NAA 0.25-4.0 mg/l). Obando and Jordan (2001) examined the potential of *in vitro* regeneration in *C. betacea*. Morphogenic responses were observed in a series of organs, mainly through somatic embryogenesis, adventitious shoots from leaf explants and root formation in axillary buds leading to plantlets. Recently, Correia *et al.* (2009) attempted to induce somatic embryogenesis from adult plants with the objective of cloning selected genotypes. However, there are no available reports on the effect of 2iP on the regeneration of plantlets from nodal explants. Furthermore, since there was no previous work on tissue culture of tamarillo in Rwanda, it was of paramount importance to evaluate the effects of different growth regulators on plant regeneration. The objectives of this study were to (i) develop an efficient, rapid propagation protocol for *C. betacea* through *in vitro* nodal culture and (ii) determine the effects of plant growth hormones on bud break and subsequent microshoot proliferation from nodal explants.

2. Materials and Methods

The nodal explants used in the study were obtained from tamarillo seedlings grown in the green house at Rwanda Agricultural Board (RAB) research station at Rubona, Rwnda. Healthy looking nodal explants selected from healthy plants were placed in a beaker containing tap water and cleaned using liquid detergent in the laboratory. Cleaned explants were then transferred to the lamina flow cabinet, immersed in 70% (v/v) ethanol for 30 seconds and rinsed twice with sterile distilled water. This was followed by surface sterilization using 10 and 15 % of NaOCl (3.85%) commercial bleach JIK®) at 15 and 20 minutes intervals. They were then rinsed 4 times in sterile distilled water. The nodal explants were cultured on MS media each supplemented with cytokinins 2 iP, kinetin and BAP (all evaluated at 5 µM/l, 10 µM/l, 20 µM/l and 40 µM/l), 30 mg/l cysteine; 100 mg/l Inositol, 3 % sucrose and gelled with 7 % agar. The Murashige and Skoog medium without growth regulators was used as control. The cultures were incubated in a growth-room maintained at 26°C and 16 hour photoperiod. All trials were established in the growth chamber using a completely randomized design with 12 replications. The data generated were analyzed using the one way analysis of variance procedure. Means separated using Tukey test (0.05) (SAS version 9.1).

3. Results

The effects of different JIK® concentrations on surface sterilization of explants at 14 days after culture are presented in Table 1. The highest proportion (92 %) of clean explants was obtained when 15 % JIK® was used for 20 minutes. The lower concentration was not effective on surface sterilization of the explants. The effects of 2iP on shoot proliferation and growth from nodal explants are presented in Table 2. 2iP at 5 µM/l gave the highest mean number (0.75±0.13) of shoots per explant and the highest mean length (25.58±5.17 mm) of microshoots. Increasing the concentration of 2iP from 5 to 20 µM/l decreased the number of shoots per node by 66.7 % and shoot length by 70.0 %. However, increasing the concentration of 2iP from 20 to 40 µM resulted in a slight increase in the number and mean length of shoots per explants.

The effects of different concentrations of BAP on shoot proliferation and growth from tamarillo nodal explants are shown in Table 3. Among all the concentrations evaluated, BAP at 40 µM/l gave the highest mean number (1.42±0.34) of shoots per explant and the highest mean length (39.25±9.05 mm) of microshoots. Increasing the concentration of BAP from 0 to 10 µM/l decreased the number of shoots per node but significantly increased shoot mean length. The number of shoots per node increased by 35.0 % and shoot length by 69.0 % when BAP concentration increased from 0 to 40 µM/l (Table 3). In general, BAP at 40µM/l was the most effective for nodal explant regeneration in tamarillo, giving rise to vigorous plants. During the current study, a one single step protocol for propagating Tamarillo was developed. The step involves culturing the nodal explant in an MS media supplemented with cytokinins (Plate 1A) induction of multiple shoots (Plate 1 B, C), elongation and rooting of microshoots (Plate 1 D, E, F) and hardening of plantlets in the green house (Plate 1 G).

The effects of Kinetin on shoot proliferation and growth from nodal explants are presented in Table 4. Kinetin at 40µM/l gave the highest mean number (1.08±0.9) of shoots per explant and the highest mean length (31.33±7.84 mm) of microshoots. Increasing the concentration of kinetin from 5 to 20 µM/l increased the number of shoots per node by 67 % and the mean shoot length by 71.0 %. Number of shoots per explant did not vary when kinetin concentration was increased from 20 to 40 µM/l but shoot length increased by 15.0 % (Table 4).

Table 1: Effects of different JIK® concentrations on elimination of surface contamination from Tamarillo nodal explants.

Concentration of JIK® (in % v/v)	Duration of immersion in JIK® (in minutes)	Contaminated explants	Percent clean explants (in %)
10	15	8	33
10	20	9	25
15	15	4	67
15	20	1	92

Table 2: Effects 2iP on shoot proliferation and growth

Concentration (μM/l)	Mean number of microshoots per explant (±SE)	Mean length of microshoots (mm) (±SE)
5	0.75±0.13[a]	25.58±5.17[b]
10	0.25±0.13[b]	8.17±4.71[a]
20	0.25±0.13[b]	7.67±4.36[a]
40	0.25±0.13[b]	12.42±6.58[a]
P value	0.0192	0.0707

Values represent means ± SE. Means within a column followed by different letters are significantly different at *P* = 0.05 (Tukey test), N = 12.

Table 3: Effects of BAP on shoot proliferation and growth

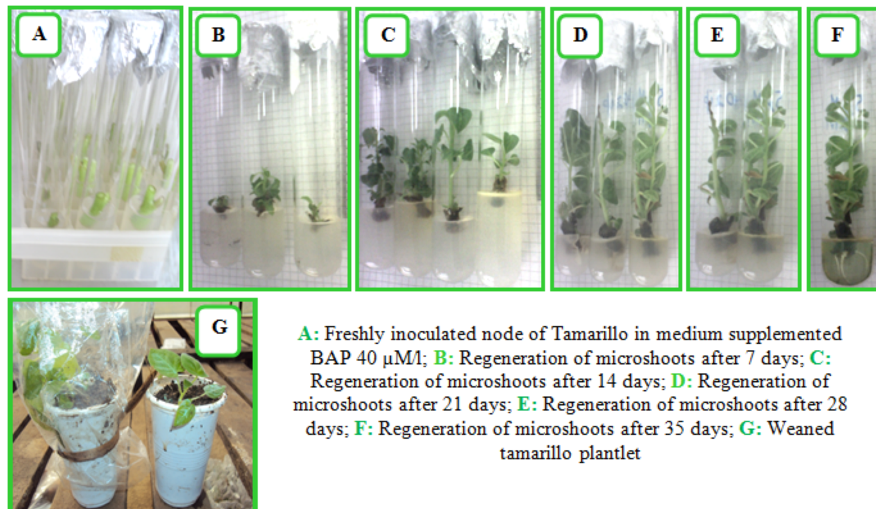
Concentration (μM/l)	Mean number of microshoots per explants (±SE)	Mean length of microshoots (mm) (±SE)
5	0.67±0.14[ba]	18.25±4.03[a]
10	0.42±0.19[b]	14.17±6.43[a]
20	0.83±0.27[ba]	19.92±7.23[a]
40	1.42±0.34[a]	39.25±9.05[b]
P value	0.0423	0.0640

Values represent means ± SE. Means within a column followed by different letters are significantly different at *P* = 0.05 (Tukey test), N = 12.

Table 4: Effects of Kinetin on shoot proliferation and growth

Concentration (μM/l)	Mean number of microshoots per explants (±SE)	Mean length of microshoots (mm) (±SE)
5	0.25±0.13[b]	7.75±4.14[b]
10	0.58±0.14[ba]	19.08±5.10[ba]
20	1.00±0.17[a]	26.58±4.88[ba]
40	1.08±0.29[a]	31.33±7.84[a]
P value	0.0142	0.0291

Values represent means ± SE. Means within a column followed by different letters are significantly different at *P* = 0.05 (Tukey test), N = 14.



A: Freshly inoculated node of Tamarillo in medium supplemented BAP 40 µM/l; B: Regeneration of microshoots after 7 days; C: Regeneration of microshoots after 14 days; D: Regeneration of microshoots after 21 days; E: Regeneration of microshoots after 28 days; F: Regeneration of microshoots after 35 days; G: Weaned tamarillo plantlet

Plate 1: Plantlet regeneration from *Cyphomandra betacea* (tamarillo) nodal explants.

4. Discussion

The success in tissue culture depends on the effectiveness of the sterilization methods used on the explants prior to culture initiation (Ankita and Handique, 2010). It is essential that all the materials used in the plant cell culture be sterilized to kill the microorganisms that are present. Contamination with microorganisms such as viruses, bacteria, yeast and fungi is the single most important reason for losses during *in vitro* culture of plants (Omamor *et al*., 2007). The disinfectants widely used are sodium hypochlorite which dates back to the mid-18th century (Miche and Balandreau, 2001). In the current study, surface sterilization was achieved using the household bleach commonly called JIK® (Reckitt and Benckiser, Kenya Ltd.) containing 3.85 % sodium hypochlorite. The use of 15 % JIK® for 20 minutes was effective, giving 92% non-infected cultures.

Plant growth regulators are the critical media components in determining the development pathway of the plant cell. The naturally occurring cytokinin 2iP and the synthetic analogues BAP and kinetin, are frequently used in plant cell culture media as they promote cell division (Adrian *et al.,* 2003). Results in the current study showed that 2ip was effective in regenerating plantlets from

tamarillo nodal explants. To the best of our knowledge, this is the first report on the successful use of 2iP to induce microshoots in tamarillo. Bertrand and Lalonde (1985) also reported that 2iP produced many shoots in similar studies in *Elaeagnus angustifolia.* BAP was found to be the best growth regulator for regenerating plantlets from nodal explants at 40 µM/l and gave the highest number (1.42±0.34) of microshoots per explant and the highest mean length (39.25±9.05 mm).

These results support those of Jafari *et al.* (2011) who reported that increasing the concentration of BAP during the initiation stage enhanced fresh weight and percentage bud formation in *Musa acuminata* cv.Berangan. Ndoye *et al.* 2003 and Vasudevan *et al*. 2001 also reported that *in vitro* shoot multiplication is a function of BAP concentration. Similarly, the importance of the application of high BAP concentration to initiate bud formation from explants were reported by Zaffari *et al*. (2000) and Subramaniam *et al.* (2008) in Cavendish banana cultivar Brasilian (AAA).

In this study, prolific rooting was achieved in all the cultures without application of exogenous auxins. This could be due to high levels of endogenous auxins in tamarillo that promoted rooting naturally in all the cultures. Another possible explanation is that cytokinins can sometimes induce or promote root growth (Fries, 1960) or adventitious root formation in the absence of auxins (Nemeth, 1979).

5. Conclusion

It was concluded from the results of the study that (i) the optimum JIK® concentration for sterilization of greenhouse grown derived tamarillo nodal explants was 15 % (v/v) for a duration of 20 minutes, (ii) optimum cytokinin concentration for maximizing root and shoot proliferation was 40 µM/l BAP and (iii) the protocol developed would enhance micro-propagation of tamarillo in Rwanda.

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References

1. Adrian, S., Nigel, S. and Mark, F. (2003). *Plant* Biotechnology: The genetic manipulation of plants. Oxford University Press, UK.
2. Ankita K and Handique P.J. (2010) Standardization of sterilization techniques prior to *in vitro* propagation of *Andrographis paniculata* (Burm.f) Nees; Asian Journal of Science and Technology. 6, 119-122.
3. Barghchi, M. (1998). *In vitro* regeneration, plant improvement and virus elimination of tamarillo [*Cyphomandra betacea* (Cav.) Sendt)]. 173-185. In: M.R. Davey *et al*. (eds.) *Tree* Biotechnology – Towards the Millennium. Nothingham University Press. Nothingham.
4. Bertrand, L.J. and Lalonde, M. (1985). *In vitro*-propagation and nodulation by Frankia of actinorhyzal Russian olive (*Elaeagnus angustifolia* L.). Plant and Soil. 87, 143-152
5. Correia, S.I., Lopes, M.L. and Canhoto J.M. (2009). Somatic embryogenesis in tamarillo (*Cyphomandra betacea*): recent advances.
6. Fries, N. (1960). The effect of adenine and kinetin on growth and differentiation of *Lupinus*. Physiol. Plant. 13, 468-481.
7. Miche, L. and Balandreau, J. (2001). Effects of rice seeds sterilization with Hypochlorite on inoculated *Burkholderia vietnamiensis*. Appl. Environ. Microbiol. 67(7): 3046-3052.
8. Ndoye M., Diallo I, and Yaye K.G. (2003). *In vitro* multiplication of the semi-arid forest tree,*Balanites aegyptiaca* (L.) Del. African Journal of Biotechnology. 2 (11), 421-424.
9. Jafari Najmeh, Rofina Yasmin, and Norzulaani Khalid. (2011). Effect of benzylaminopurine (BAP) pulsing on *in vitro* shoot multiplication of Musa acuminata (banana) cv.Berangan. African Journal of Biotechnology. 10(13),2446-2450.
10. Nemeth, G. (1979). Benzyladenine-stimulated rooting in fruit-tree rootstocks cultured *in vitro*. Z. Pflanzenphysiol. 95, 389-396.

11. Obando, M. and Jordan, M. (2001). Regenerative responses of *Cyphomandra betacea* (Cav.)
12. Sendt. (tamarillo) cultivated *in vitro*. Acta Horticultura. 560: 429-432.
13. Omamor, I. B., Asemota, A.O., Eke, C.R. and Ezia, E.I. (2007). Fungal contaminants of the oil palm tissue culture in Nigerian Institute for Oil Palm Research (NIFOR). Afr. J. Agric. Res. 2(10): 534-537.
14. Subramaniam, S., Rathinam, X., Poobathy, R. and Sinniah, U. (2008). *In Vitro* production of multiple bud clumps (Mbcs) from Cavendish Banana Cultivar, Brasilian (AAA). American-Eurasian J. Sustainable Agric. 2(3): 300-307.
15. Vasudevan, A., Selvaraj N., Sureshkumar, P. and Ganapathi, A. (2001). Multiple Shoot Induction from the Shoot Tip Explants of Cucumber *(Cucumis sativus L.)* Department of Biotechnology, School of Life Sciences, Bharathidasan University, Trichirappalli-620024, Tamilnadu, India.
16. Zaffari, G.R., Kerbauy, G.B., Kraus, J.E. and Romano, E.C. (2000). Hormonal and histological studies related to *in vitro* banana bud formation. Plant Cell Tissue Organ Culture. 63(3): 187-192.