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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Induction and regeneration of somatic embryos from *Vitex doniana* (Lamiaceae) leaf explants

Colombe Dadjo[1], Jane Kahia[2]*, Catherine Muthuri[3], Lucien Diby[2], Christophe Kouame[2], Peter Njenga[4] and Modeste Kouassi[5]

[1]Institute of Basic Sciences, Technology and Innovation, Pan African University, P. O. Box 62000-00200, Nairobi, Kenya.
[2]World Agroforestry Centre (ICRAF), Cote d' Ivoire Country Program Cocody Mermoz, Abidjan, Côte d'Ivoire.
[3]ICRAF World Agroforestry Centre (ICRAF), P. O. Box 30677-00100, Nairobi, Kenya.
[4]Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.
[5]Centre National de Recherche Agronomique (CNRA), Laboratoire Central de Biotechnologies (LCB), 01 BP 1740 Abidjan 01, Côte d'Ivoire.

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The present study was conducted with the aim of evaluating some of the factors that influence induction and regeneration of somatic embryos in *Vitex doniana* since there are no available reports on tissue culture of this tree species. Leaves from plants growing under temporary shed were cultured on Murashige and Skoog supplemented with silver nitrate and four amino acids (proline, tryptophan, lysine and leucine) at varying concentrations, 0.11 mg/l thidiazuron, 2% sucrose and 100 mg/l myo-inositol in separate experiments. The explants cultured on media supplemented with tryptophan at 30.6 mg/l produced the optimal (6.5) number of embryos per explant. This number was fivefold more than the number obtained in the control. On the other hand, it was observed that the explants on media supplemented with silver nitrate at 8.45 mg/l gave the same mean (6.5) number of embryos per explant. These first ever results on the induction of somatic embryo in *V. doniana* could be used for mass propagation and to select useful traits of this tree species at the cellular level. However, further work needs to be done on the conversion of the regenerated embryos.

Key words: Amino acids, leaf explant, silver nitrate, somatic embryogenesis, *Vitex doniana*.

INTRODUCTION

Many neglected and underutilized wild species (NUS) are nutritionally rich (Ghane et al., 2010; Johns and Eyzaguirre, 2006). Therefore, their erosion can have immediate consequences on the nutritional status and food security of the poor and their enhanced use can bring about better nutrition and fight hunger. Even though the link between agrobiodiversity and diet diversity is not automatic (Burchi et al., 2011), it is agreeable that the diminution of

*Corresponding author. E-mail: j.kahia@cgiar.org.

agrobiodiversity, to some extent, places considerable strain on the ease with which households are able to enjoy diversified, balanced diets. Awareness of the importance and value of crop wild relatives and of the need to conserve them has increased one of such high value plant that is in dire need for conservation, *Vitex doniana* Sweet (Black plum). It had been considered to belong to Verbenaceae family by different authors but in recent works, it has been transferred into Lamiaceae based on different evidences (Wagstaff et al., 1998). It is the most abundant and widespread *Vitex* species in Africa (Orwa et al., 2009). Black plum (*V. doniana)* is an indigenous species important for the livelihoods of rural populations in Benin Republic in particular and West Africa in general (Codjia et al., 2003). The fruits and leaves are the edible part of the trees. They are either eaten raw or after processing. The plant is also widely used in traditional system for medicinal purposes. The leaves, fruits, roots, barks and seed of the plant have been used as medication for liver disease, anodyne, stiffness, leprosy, backache, hemiplegia, conjunctivity, rash, measles, rachitis, febrifuge, as tonic galactagogue to aid milk production in lactating mothers, sedative, digestive regulator, treatment of eye troubles and kidney troubles. It has also been used for treatment of conditions such as infertility, anemia, jaundice, dysentery, gonorrhea, headaches, diabetes, chickenpox, rash and fever (Louppe et al., 2008; Orwa et al., 2009). Despite the widely known nutritional, medicinal and economic uses of *V. doniana* products, the species is still under-utilized, unimproved. To date, the species has been chosen as a model species to be domesticated in Benin (Codjia et al., 2003; Dadjo et al., 2012).The strong anthropic pressure affecting this species has caused its numbers to fall increasingly in its natural environment (Achigan-Dako et al., 2010). The planting of seedlings is negligible and the seeds of this tree have a very weak germinating capacity (Thies 1995). Sanoussi et al. (2012) reported that macropropagation rate of this tree species by stem cuttings is slow. Therefore, there is need to seek for alternative propagation methods. Tissue culture methods are considered as the most promising means to protect and propagate tree species of economic interest. *In vitro* propagation through somatic embryogenesis is the most feasible alternative to the other *in vitro* methods (Zimmerman, 1993, Saiprasad, 2001). Somatic embryos are widely considered to be of single cell origin; hence this is advantageous for transformation studies. Moreover, the process of somatic embryogenesis offers a mean to propagate large numbers of transgenic plants over a short period of time.

The success in tissue culture depends on the effectiveness of the sterilization methods used on the explants prior to culture initiation (Yildiz and Celal, 2002). Sterilization is the process of eliminating contamination from explants before establishment of *in vitro* cultures. Various sterilization agents such as calcium hypochlorite and sodium hypochlorite are commonly used to decontaminate the tissues.

Ethylene is known to reduce somatic embryogenic competence in many plants and the use of silver nitrate ($AgNO_3$), an ethylene action inhibitor (Beyer, 1976), has been shown to influence *in vitro* somatic embryogenesis (Kong et al., 2012). It has also been reported that amino acids play a key role in plant growth and development because they are good source of nitrogen (Kirby et al., 1987; Shanjani, 2003). Amino acids such as glutamine, proline and tryptophan, have been identified as enhancers of somatic embryogenesis in some species (Deo et al., 2010). Their efficacy in embryogenesis has been attributed to their contribution to various cellular processes such as improving cell signaling processes in various signal transduction pathway (Lakshmanan and Taji, 2000) and as precursor molecules for certain growth regulators. So far, there are no reports on tissue culture of *V. doniana.* During the current study, we hypothesized that the various concentrations of $AgNO_3$ and some amino acids could enhance induction and regeneration of somatic embryos in *V. doniana.* Therefore, the aim of this study was to evaluate the effects of various concentrations of $AgNO_3$ and some amino acids on induction and regeneration of somatic embryos in *V. doniana*.

MATERIALS AND METHODS

V. doniana seedlings (2-4 years old) originally wildings collected from the field in Glo in the south part and Cove in the central part of Benin were transported to Abidjan, Côte d' Ivoire where they were maintained in a temporary shed and watered daily for one month before harvesting the leaves.

Explant preparation and surface sterilization

Healthy looking young leaves (2^{nd} pair) were collected and cleaned with cotton wool soaked with liquid soap. Thereafter, they were immersed in 0.5% (w/v) fungicide (Ridomil) containing two drops of Tween-20 (wetting agent) for an hour. The leaf explants were then transferred to the lamina flow cabinet for surface sterilization. Pre sterilization was carried using 70% ethanol solution for thirty seconds and then rinsing twice with sterile distilled water. The explants were subjected to further sterilization using varying (1, 1.5 and 2%) concentrations of calcium hypochlorite ($CaOCl_2$) containing 2-3 drops of Tween 20 and varying time duration. In an attempt to increase the number of clean explants, double sterilization (two steps) was conducted. This involved sterilizing the explants using 2% calcium hypochlorite for 30 min rinsing twice with sterile distilled water followed by quick dip in 70% ethanol. They were then subjected to a second step, by sterilizing them with 2% calcium hypochlorite for 15 min and finally rinsing with sterile distilled water four times.

Media preparation and culture conditions

Silver nitrate (at concentrations 8.45; 16.9 and 25.35 mg/l) and four amino acids namely proline (5.75; 11.5 and 17.25 mg/l), tryptophan (10.2; 20.4 and 30.6 mg/l), lysine (7.3; 14.6 and 21.9 mg/l) and leucine (6.55; 13.1 and 19.65 mg/l) were added to half strength

Table 1. Effect of $CaOCl_2$ on elimination of surface contamination from *V. doniana* leaves explants.

Concentrations of calcium hypochlorite (in % w/v)	Exposure time (min)	Percentage of clean explants (%)
1	25	0
1	30	0
1	35	0
1	40	0
1.5	20	0
1.5	25	0
1.5	30	0
1.5	35	20
2	15	40
2	20	70
2	25	70
2	30	70

Table 2. Effect of different $AgNO_3$ concentrations on induction and regeneration of somatic embryos in *V. doniana.*

$AgNO_3$ conc. (mg/l)	Embryogenic cultures (%)	Mean number of embryos/explant ± SE
0	91	1.2 ± 0.13b*
8.45	90	6.5 ± 0.41a
16.9	67	1.5 ± 0.27b
25.35	25	1.75 ± 0.25b
P-value		0.000

*Means followed by the same letter are not significantly different at p > 0.01.

Murashige and Skoog (MS) (1962) medium supplemented with 100 mg/l myo-inositol, 0.11 mg/l TDZ and 2% sucrose in separate experiments. The pH of the medium was adjusted between 5.7 and 5.8 prior to the addition of the solidifying agent, and autoclaved at 121°C for 15 min. The MS medium without $AgNO_3$ and amino acids is referred to as control. All cultures were incubated in a dark room maintained at 25 ± 2°C.

Experimental design, data collection and analysis

Completely randomized design was used for all the experiments. Each single explant was considered as experimental unit. Twenty (20) replicates per treatment were used at the outset of experiments and each treatment repeated at least twice. The data were subjected to one-way analysis of variance (ANOVA) and the significant differences between treatment means were assessed by using Minitab version 14 Software. The results of the sterilization experiment are expressed as percentage (%) while for regeneration of somatic embryos data are presented as means ± standard error (SE).

RESULTS

Surface sterilization

The highest percentage (70%) of clean leaf explants were obtained when 2% calcium hypochlorite was used for 20, 25 and 30 min, respectively (Table 1). However, this percentage decreased further and after two weeks only 50% clean explants were observed. The evaluation of double sterilization treatment resulted in increase in percent clean explants from 50 to 91% after one month and this procedure was used for all the subsequent experiments.

Effect of $AgNO_3$ on induction and regeneration of somatic embryos

The addition of $AgNO_3$ to the culture medium had a significant effect on the mean number of embryos per explant (Table 2). The percent embryogenic cultures decreased with increasing $AgNO_3$ concentration. The highest (91 and 90 %) frequency of embryogenic cultures were observed in the control and in the media supplemented with 8.45 mg/l $AgNO_3$. The later $AgNO_3$ concentration produced the highest (6.5) mean number of embryos. On the other hand, the lowest (1.2) mean number of somatic embryos was produced in the control. Significant differences were detected between the number of somatic embryos per explant at 8.45 mg/l

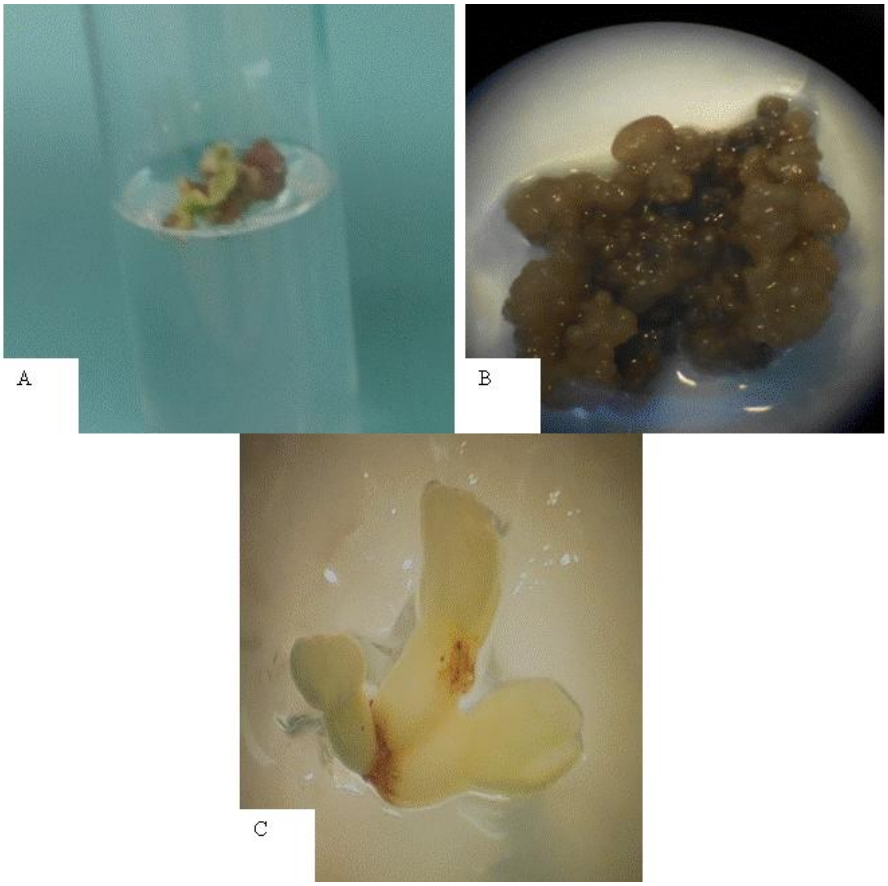

Figure 1. A) Callus, B) globular embryos, C) cotyledonary embryos.

$AgNO_3$ and the other concentrations of $AgNO_3$ at $p < 0.01$. Callus was observed from the cut edges of the leaf and globular embryos were obtained four weeks after culture (Figure 1A and B). Cotyledonary stage of embryos was observed two months after culture as shown in Figure 1C.

Effect of some amino acids induction and regeneration of somatic embryos

Among the four amino acids evaluated, the highest leucine concentration (19.65 mg/l) was found to inhibit embryo formation (Table 3). However, when leaf explants were cultured on media supplemented with the lower concentrations (6.55 and 13.1mg/l), the mean number of embryos obtained were double those obtained in the control. It was observed that increasing the concentration of proline from 5.75 to 17.25 mg/l decreased significantly the mean number of embryos. Leaf explants cultured on medium supplemented with 30.6 mg/l tryptophan produced the highest (6.5) mean number of somatic embryos which was fivefold more than the embryos obtained in the control.

DISCUSSION

The current study was conducted with the aim of evaluating some of the factors that influence induction and regeneration of somatic embryos since there were no available reports on tissue culture of *V. doniana.* Before any explants is placed into culture, it is essential to destroy all microorganisms and the success in tissue culture depends on the effectiveness of the sterilization methods used on the explants prior to culture initiation (Yildiz and Celal, 2002). The outer surface of plants growing under natural or greenhouse conditions is normally infected with spores and other microbial cells. The use of field grown plants as a direct source of explant material for obtaining ‘clean’ explant, presents a major challenge. Previous attempts to initiate clean explants from field grown coffee, especially those from canopy close to the ground, resulted in 100% contamination (Kahia, 1999). In an attempt to obtain clean *in vitro* cultures, sources of contamination other than surface contaminants need to be considered. Even if the surface of the explant is effectively sterilized, the contaminants could emanate from the inner tissues when the plant materials are dissected into small explants after

Table 3. Effect of different amino acid concentrations on induction and regeneration of somatic embryos in *V. doniana.*

Amino acid	conc. (mg/l)	Embryogenic cultures (%)	Mean number of embryos/explant ± SE
Control	0	91	1.2 ± 0.13a*
	6.55	25	2.67 ± 1.2b
Leucine	13.1	50	2.4 ± 0.51b
	19.65	0	0[x]
	7.3	30	1.33 ± 0.21a
Lysine	14.6	38	1.5 ± 0.34a
	21.9	50	1.6 ± 0.4a
	5.75	10	3.5 ± 0.5b
Proline	11.5	10	2.0 ± 0.0a
	17.25	6	1.5 ± 0.5a
	10.2	42	2.33 ± 0.21b
Tryptophan	20.4	17	1.5 ± 0.5a
	30.6	60	6.5 ± 0.66 c
P-value			0.000

x. The zero response is not included in the statistical analysis. Means followed by the same letter are not significantly different at $p > 0.01$.

the surface sterilization. Systemic contaminants, for example, are not eliminated by surface sterilization (Webster et al., 2003). For this reason, a systemic fungicide such as Ridomil used in this study can be helpful to control the incidence of internal fungal infection in explants. The disinfectant widely used for surface sterilization of explants in tissue culture is sodium hypochlorite which dates back to the mid-18th century (Miche and Balandreau, 2001). It is usually purchased as household laundry bleach and as such it is readily available and can be diluted to proper concentrations. A balance between concentration and time must be determined empirically for each type of explant because of phytotoxicity. Previous reports indicate that bleach (1.4% NaOCl solution for 1 min) was found to be effective in sterilizing greenhouse-derived leaf explants in *Aquilaria crasna* and *Aquilaria sinensis* (Okudera and Ito, 2009). On the other hand, effective sterilization was achieved by using 50% bleach for 20 min on shoot tips of *Aquilaria hirta*, from greenhouse (Hassan et al., 2011) while leaf explants of *Allanblackia stuhlmannii* were best surface sterilized using 8% sodium hypochlorite (Neondo et al., 2011). The other sterilant used for decontaminating explants is calcium hypochlorite. It is known to be less injurious to plant tissues and is generally used at a concentration of 3.25% (CSS 451 2009). During the present study, calcium hypochlorite was used as attempts to use the commercial bleach even at very low concentrations was found to be phytotoxic and led to the death of explants. The lower concentrations of calcium hypochlorite were not effective in decontaminating the explants. These results concur with those of Mihaljević et al. (2013) who reported high percent sterilization (80%) of sour cherry nodes when a high (3%) concentration of $CaOCl_2$ was used. A two-step sterilization was adopted in the work being reported in order to increase the percent clean explants and using this procedure, 91% clean explants was recorded. Similar results were reported by Nieves and Evalour (2011) who recorded 90% clean explants from cotyledon of *Moringa oleifera* using two steps comprising of 5% $Ca(OCl)_2$.

There are conflicting reports on the use of silver nitrate in induction and regeneration of somatic embryos. When added at low concentrations of 5 to 50 µM (0.845 to 8.45 mg/l), it was found to inhibit somatic embryo formation in *Coffea canephora* leaf explants (Hatanaka et al., 1995). On the other hand, media supplemented with $AgNO_3$ has been shown to improve somatic embryogenesis in species such as *Triticum durum* (Poaceae) (Fernandez et al., 1999), *Coffea canephora* (Rubiaceae) (Fuentes et al., (2000), *Spinacia oleracea* (Ishizaki et al., 2000), *Carthamus tinctorius* (Asteraceae) (Mandal et al., 2001), *Paspalum scrobiculatum* (Poaceae) (Vikrant, 2002), *Bactris gasipaes* (Arecaceae) (Steinmacher et al., 2007), *Paspalum scrobiculatum* (Poaceae) and *Eleusine coracana* (Poaceae) (Kothari-Chajer et al., 2008), *Hedychiurn bousigonianum* (Gingeberacea) (Sakhanokho et al., 2009), *Gossypium nelsonii* (Malvaceae), *Gossypium australe* (Yan et al., 2010) and *Pinus taeda* (Pinaceae) (Pullman et al., 2003). Kong and Yeung (1995) reported that 100 µM $AgNO_3$ (16.9 mg/l $AgNO_3$) stimulated embryo formation in white spruce. The exact mechanism by which $AgNO_3$ affects somatic embryogenesis is not completely understood (Kong et al., 2012). During the current study, incorporating 8.45 mg/l silver nitrate in the media led to a fivefold increase in the

number of embryos as compared to the control. However, it was observed that increasing the concentration from 8.45 to 25.35 mg/l decreased both the percentage of embryogenic cultures and the mean number of embryos. These results are contrary to those of Kong et al. (2012) who reported that the number of somatic embryos per explant in Manchurian ash increased with increasing $AgNO_3$ concentration. These workers reported that the lowest number of embryos per explant (1.0) was induced at 2.5 mg/l $AgNO_3$ while the higher concentration of 10 mg/l resulted to a threefold increase in the number of somatic embryos with mean (3.86) embryos per explant. Comparable results have also been reported by Ishizaki et al. (2000) in *Spinacia oleracea* where addition of 10 mM $AgNO_3$ (1.69 g/l $AgNO_3$) to the medium resulted in formation of about three times more embryos as compared to the controls. Fuentes et al. (2000) found that the addition of $AgNO_3$ caused only small modifications in the ionic equilibrium of the medium and concluded the effects of the compound on somatic embryogenesis were not attributable to any substantial changes in available nutrients.

Requirement of exogenous supply of amino acids for *in vitro* somatic embryogenesis has been reported in a number of plant species (Basu et al., 1989; Claparols et al*.,* 1993). For instance, glutamine was found to be beneficial for embryo development in date palm (El-Shiaty et al., 2004). These workers reported that MS medium supplemented with 100 mg/l glutamine gave the highest (3.33) mean number of embryos. In another report, incorporating proline in sugarcane cultures enhanced somatic embryogenesis (Gill et al., 2004). In *Peucedanum oreoselinum,* embryo formation and maturation was enhanced by addition of proline in MS medium (Coste et al., 2011). On the other hand, in wheat culture, the efficiency of the amino acids was found to be genotype-based (Duran et al., 2013). Sarker et al. (2007) reported that there were significant responses when L-asparagine at 150 mg/l concentration was used in four popular Bangladeshi wheat cultivars *viz* Kanchan, Shourav, Gourav and Satabdi. In the work being reported, it was found that supplementing MS media with amino acid promoted the induction and regeneration of somatic embryos. These results concur with those of Shahsavari (2011) who reported that when 100 to 300 µM (20.4 to 61.2 mg/l) tryptophan was added to callus induction medium of rice, there was a great enhancement of the frequency of embryogenic cultures and regeneration of somatic embryos. The lowest concentration of proline evaluated in the current study increased almost three folds the mean number of embryos as compared to the control. However, increasing the proline concentration resulted in decrease in the frequency of embryogenic cultures and the mean number of embryos. These results are contrary to those reported by Chowdhry et al. (2003) who observed that increasing the concentration of proline enhanced embryogenesis in rice. Differential responses of different amino acids indicate the requirement of specific amino acids for somatic embryo regeneration in *V. doniana*.

Conclusion

This study clearly demonstrates that this tree species is amenable to somatic embryogenesis. The results of the current study will be a valuable tool to complement the production of planting materials and thus help in exploiting the medicinal and nutrition value of this tree for the rural poor in Benin and Africa in general. *Ex situ* and domestication programs of *V. doniana* could benefit from the findings of the current study. There is however need to do more work on the conversion of somatic embryos.

Conflict of interests

The authors hereby declare that there is no conflict of interest.

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