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The Asian Journal of Animal Science (December 2009 to May 2010) Vol. 4 Issue 2 : 231-236

RSEARCH PAPER

Improvement of secondary metabolites by somatic hybridization of *Murraya koenigii* and *Centella asiatica*

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Accepted : November, 2009

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ABSTRACT

Somatic hybridization may be a useful tool for the genetic modification of chemical composition of the hybrid plant. This study was designed to improve the secondary metabolite by somatic hybridization of medicinally important plants *Murraya koenigii* and *Centella asiatica.* The protoplasts were isolated by enzymatic method and they were fused with PEG. The hybrid protoplasts were selected using drug sensitivity method (Tetracycline). For establishing the microcalli B5 medium enriched with BA 1.00 mg/l,BA 2.00 mg/l showed higher activity. The combination of BA and NAA or IAA at different levels induced callus. The methanolic extracts of hybridized callus and parental plants were prepared. Secondary metabolites produced were seperated by TLC and compared by their obtained Rf value and antibacterial activity. The work showed fused callus extracts with higher Rf value and also showed more antibacterial activity than the parental extracts, however the study is a stepping stone for improvement of secondary metabolite.

Key words : BA, IAA, NAA, Kinetin, TLC, Somatic hybridization,PEG

Plants are the source of many of our most important pharmaceuticals. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches plant tissue culture are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites.(Ramachandra Rao and Ravishankar 2002). Plant secondary metabolism is the way to successful applications in molecular farming, health food, function food, plant resistance and somatic hybridization show enormous potential for the genetic engineering of plant secondary metabolism. Somatic hybridization also provides the possibility of producing hybrids with improved combination of secondary metabolite.(Lou.*et al*.,1999) and is an important tool for genetic improvement programs (Mendes-da-Gloria *et al*., 2009) and has a great importance in species transfer and genomic projects(Gonter *et al*., 2002). Grezes *et al*., 1994 reported that the yield of secondary metabolite production about 10 to 15 fold by preparing single clones, using protoplast system of *Coffea arabica*.

In the present study, the medicinal plants *Centella asiatica* (Vallarai) and *Murraya koenigii* (Curry leaves) were selected as the parental plants. *Centella asiatica* is a mildly antibacterial, antiviral, anti inflammatory, anti ulcerogenic anxiolytic, a cerebral tonic, a circulatory stimulant, a diuretic and vulnerary. The major secondary compounds are Centellin, Asiaticin, and Centellicin (Siddiqui *et al*., 2007).

The medicinal properties of *Murraya koenigii* include antidiabetic, antioxidant, antimicrobial, anti-inflammatory, hepato protective and anti-hypercholesterotemic. The major compounds include caryophyllene, sabinene, α and β pinene, terpenes, humulene (Rana *et al*., 2004). Both the parental plants having antimicrobial and antioxidant activity (Gupta *et al*., 2009). So far there is no report regarding the somatic hybridization of these two plants.Hence this study was mainly focused on the production of somatic hybrids using protoplasts fusion of *Centella asiatica* and *Murraya koenigii* for improvement of secondary metabolites.

MATERIALS AND METHODS

Protoplast isolation and fusion:

The young leaves of parental plants were collected from the field for the isolation of protoplast. The explants were surface sterilized with 0.08% mercuric chloride for 12 minutes,and they were washed with sterile distilled water. The protoplasts were isolated by mixed enzymatic method (Power and Cocking 1970). The enzymes solution contained cellulase onozuka R-10 (0.05,0.1and0.2%) and Pectinase (0.1,0.2and0.3%) and the enzyme mixture filter sterilized through 0.2µm membrane filters (Table 1). Observations taken were based on the time taken for the

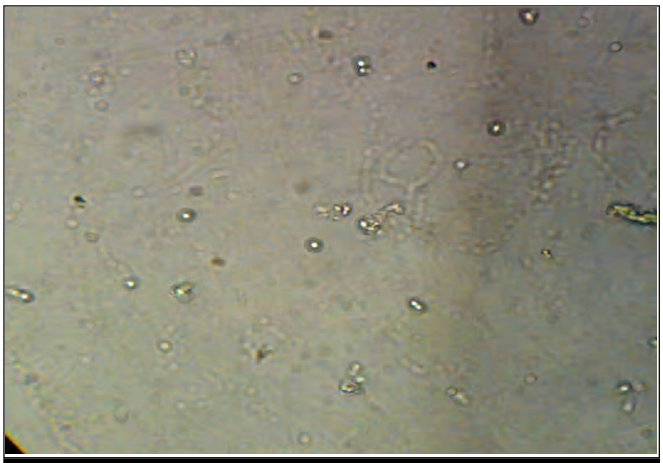

Fig. 1 : Microscopic photograph showing enzymatically released protoplasts

isolation of protoplast. The viability of protoplasts was assessed by using phenyl safranin. Then isolated protoplasts were purified by centrifugation and then fused with the fusagens like PEG (56%) and $CaCl_2$ (pH 10) and after fusion, the fused protoplasts were selected using the drug sensitivity method. (Power *et al.,* 1976)

Culturing of hybrid cells :

For estabilishing microcalli, the selected hybrids derived microcolonies were subcultured in both liquid and solid basal Gamborg B5 medium (Gamborg *et al*., 1968) fortified with 1.00,2.00,3.00 mg/l BA and the combination of IAA 2.00+BA 2.00, NAA 0.5+KN 1.00, NAA 1.00+ KN 1.00 mg/l.(Table 1). And the microcalli developed were transferred to basal B5 medium fortified with 0.5, 1.00, 1.50, 2.00 mg/l BA and combination of BA 1.00 mg/ l and IAA 0.5,1.00 mg/l,NAA 0.5.1.00 mg/l. (Table 2)

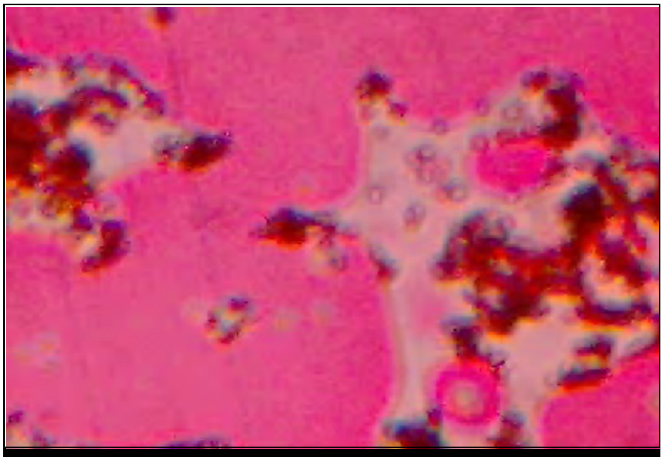

Fig. 2 : Protoplast stained with phenyl safranin showing dead protoplasts as red and viable cells as colourless

Comparative analysis :

The methanolic extract of parental plants and the fused callus extract were utilized for the comparative analysis.20 grams of dried powder mixed with 250 ml of methanol,and the extraction was carried out at specific boiling temperature of solvent using soxlet apparatus for 48 hours for complete extraction of secondary compounds The final filtrate were concentrated using Vaccum drier and can be used for further experiments.

The calli were retrieved from the suspension culture and dried in hot air oven at 60^0C for 24 hours. The fresh and dry weights of calli was recorded. For extraction the powdered calli was put in 80% methanol and kept in shaker. After 5 days, the extract was filtered through Whatmann (No. l) filter paper to get the filtrate. The methanol was added in the proportion to get a concentration of 10 mg/ml.

Secondary metabolite production from the natural parental plant and the invitro hybridized callus were compared using antibacterial assay (Test microorganisms include *Streptococcus mutans, Bacillus megatherium, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia*) and by thin layer Chromatography (Silica Gel was used as a stationery phase, the mixture of propanol and Glacial acetic acid in the ratio of 99:1) was used as the mobile phase.

RESULTS AND DISCUSSION

In the present study protoplast of *M.koenigii and C.asiatica* were successfully isolated from leaf using the enzyme (cellulase and pectinase) mixture. The effects of different combinations of enzyme mixtures on the yield of isolated protoplast were presented in Table 1. The enzyme mixture (cellulase 0.1+ pectinase 0.1%) produced the highest yield in both plants (*M.koenigii* -45 nos and *C.asiatica*-51 nos) with in 2 hours of incubation. About 60 % of viable cells were observed in this treatment. All other treatments were found to be almost of same effect. After purification different fusagens like PEG and

Table 1: Effect of enzyme concentrations on the isolation of protoplasts

Treatments	Pectinase (%)	Cellulase (%)	Number of protoplast isolated		Time (hours)
			Mk	Ca	
T_1	0.1	0.05	22	26	3
T_2	0.1	0.1	45	51	2
T_3	0.1	0.2	30	37	3
T_4	0.2	0.2	26	30	4
T_5	0.3	0.2	20	28	3

calcium chloride were tried to study the effect on fusion. Among these, PEG (Poly ethylene glycol) was found to be good for the fusion of protoplast. The fused protoplast were selected by using drug sensitivity method. In the the parental protoplast failed to multiply on the medium fortified with tetracycline and the hybrid protoplast was able to survive and produces micro colonies in the presence of tetracycline. The cultured protoplast showed the first cell division after 48 hours of culture and developed in to cell colonies after 15 divisions. The selected micro colonies were sub cultured in both liquid and solid based medium supplemented with various level of plant growth regulator for the development of micro calli. Basel media with BA alone was found to be good for the production of micro colony. BA at lower concentration produces more number of colonies (200 number) compared with others. No colony formation was observed in the combination of NAA and kinetin. Suspension culture showed earlier colony formation compared with solid medium (Table 2, Fig. 3). These cell colonies were transfered to basal medium enriched with various levels of plant growth regulators for the induction of micro calli. Of these mixture of BA and IAA (1.00, 2.00 mg/l) produced small creamy white callus with in 17 days. The combined form of BA (1.00mg/l) and NAA (2.00 mg/l) produced powdery callus with in 15 days of incubation. No callus formation was observed in lower concentration of BA (0.5, 1.00, 1.5 mg/l).Callus formation occured after 22 days of incubation at BA 2.00 mg/l (Table 4, Fig. 4). The fused callus extract and the parental plant extract were subjected to TLC analysis and anti microbial studies for the comparative evaluation. The hybridized callus showed higher Rf value (0.8cm) compared with parental plants (Table 4, Fig. 6).The Rf value of the parental extract were 0.70 cm in *M.koenigii* and 0.73 cm in the case of *C.asiatica* respectively. The results of antibacterial activity showed that the crude extracts of hybridized callus have higher antibacterial activity compared with parental plant extracts (Table 5, Fig. 5).The hybrid showed higher activity against *Proteus vulgaris* and *Klebsiella pneumonia*.The zone of inhibition was 13 and 10 mm, respectively. C. *asiatica*

Table 2 : Effect of plant growth regulators on colony formation

Treatments	Plant growth regulators	Number of microcalli
H_1	BA 1.00	200
H_2	BA2.00	150
H_3	BA3.00	112
H_4	IAA2.00+BA2.00	26
H_5	NAA0.5+KN1.00	No colony formation
H_6	NAA1.00+KN1.00	No colony formation

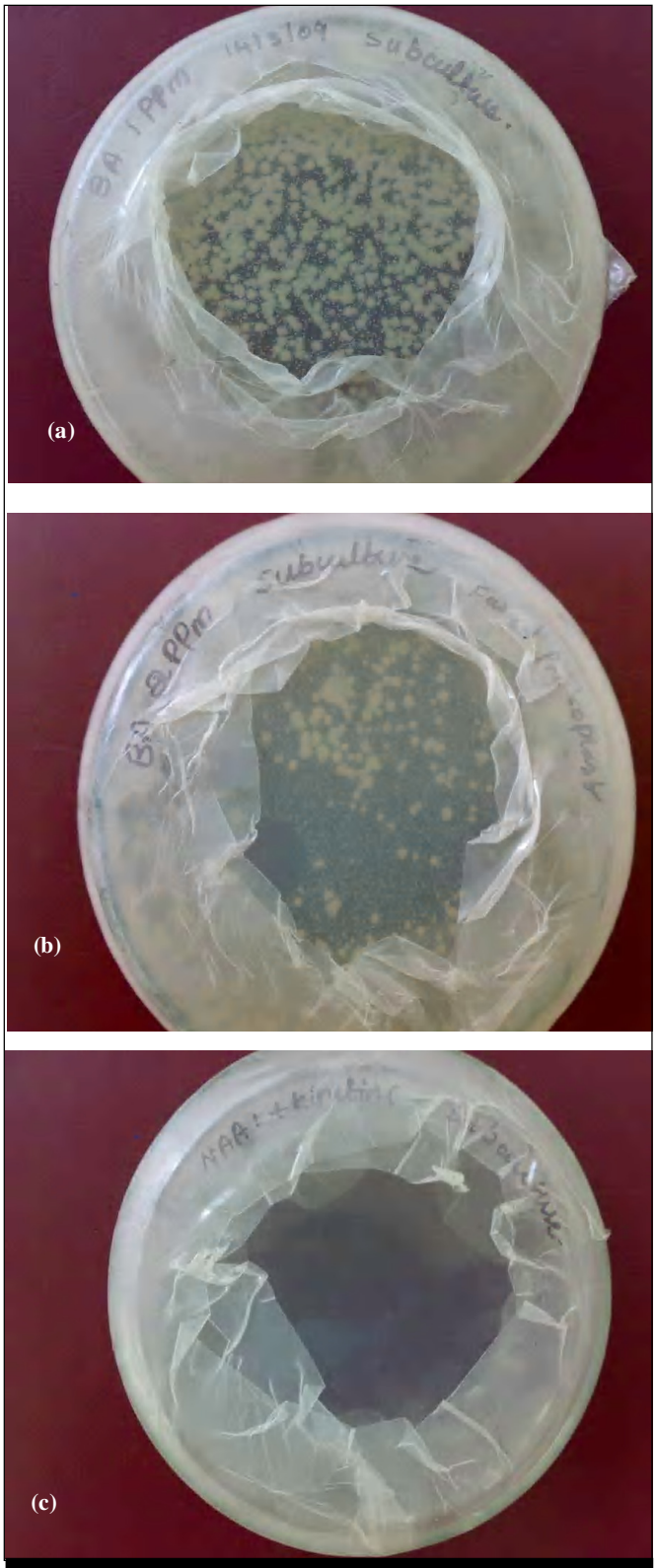

Fig. 3 : Culturing of hybrid cells
a) Formation of microcalli in B5 solid medium supplemented with BA 1.00 mg/l
(b) Formation of microcalli in B5 solid medium with BA 2.00 mg/l
(b) Formation of microcalli in B5 solid medium with BA 2.00 mg/l

Table 3 : Effect of plant regulators on callus production

Treatment	Plant growth regulators	Number of days taken	Nature of callus
H_1	BA 0.5	No callus formation	-
H_2	BA 1.00	No callus formation	-
H_3	BA 1.50	No callus formation	-
H_4	BA 2.00	25	Small powdery callus
H_5	BA 1.00+IAA 1.00	17	Small creamish white callus
H_6	BA 1.00+IAA 2.00	22	Creamish white callus
H_7	BA1.00+NAA1.0	15	Creamish white powdery callus
H_8	BA1.00+NAA2.00	13	Creamish white compact callus

Basal medium : B5 major + MS minor +Sucrose 30 g/l+ Inositol 100 mg/l

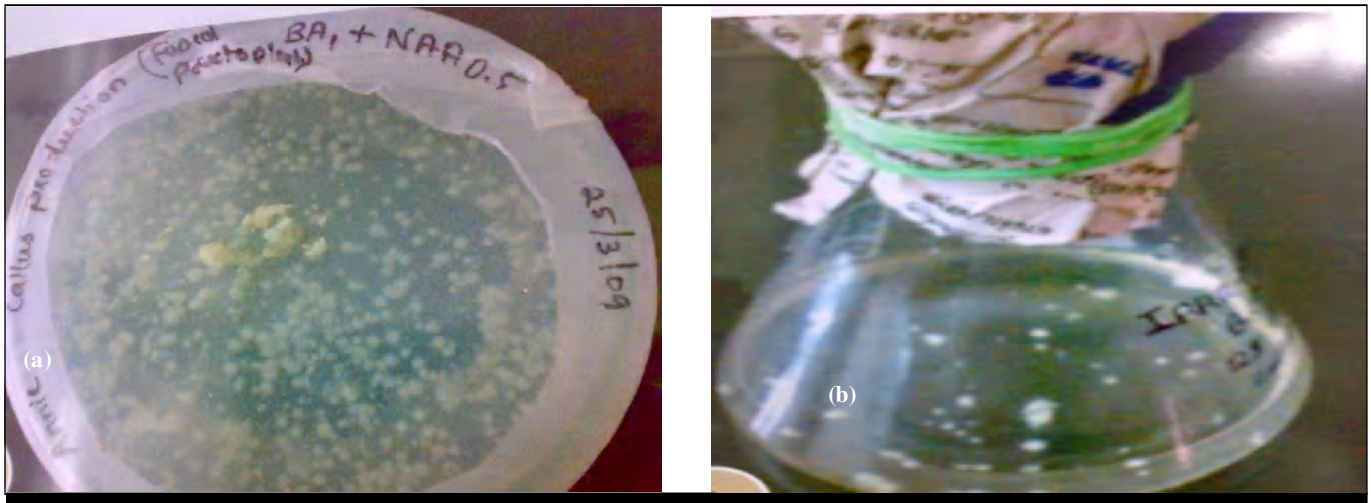

Fig. 4 : Callus production
(a) Formation of creamish white powdery callus in B5 medium
(b) Formation of small creamish white callus in suspension culture

Table 4 : The TLC result shows the Rf value

Extracts	Rf value (cm)
Fused callus (T_1)	0.8
Centella asiatica (T_2)	0.73
Murraya koenigii(T_3)	0.70

showed higher activity against *proteus vulgaris* (10 mm) and the *M.koenigii* was found to be more active against *Streptococcus mutants*. The zone of inhibition was 12mm in diameter

Protoplast technology has been considered as an important and valuable approach to produce noval genotypes with desired traits through parasexual hybridization, genetic engineering and somaclonal variation. Protoplast fusion provides a unique opportunity for the genetic improvement of medicinal species(Azad *et al*.,2006).In the present study young leaves of plants were used as the source of protoplast. Leaf is the best source of protoplast because the mesophyll cells are

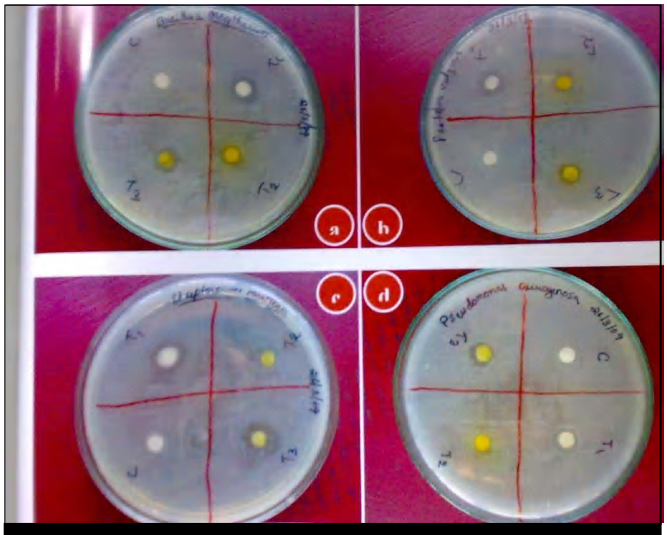

Fig. 5 : Antibacterial assay
a-*Bacillus megatherium*
b-*Proteus vulgaris*
c-*Streptococcus mutants*
d-*Pseudomonas aerogenosa*

Table 5 : Antimicrobial activity

Sr. no.	Bacterial strains	Zone of onhibition (mm)		
		Fc	*C.asiatica*	*M.koenigii*
1	*Streptococcus mutants*	9	8	12
2	*Bacillus megatherium*	9	7	7
3	*Pseudomonas aerogenosa*	10	9	8
4	*Proteus vulgaris*	13	10	8
5	*Klebsiella pneumonia*	7	8	7

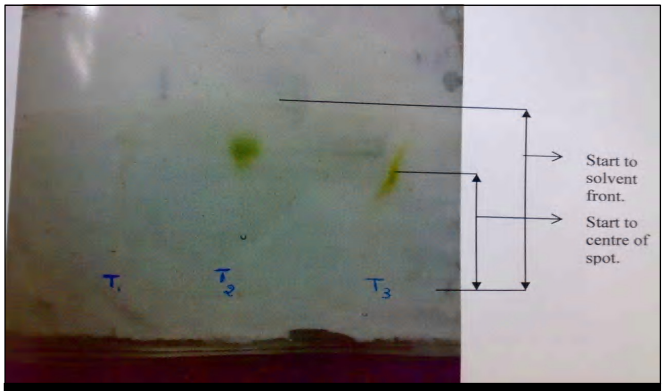

Fig. 6 : Thin Layer chromatography
T_1- Fused callus
T_2- *Centella asiatica*
T_3- *Murraya koenigii*

loosely arranged. So the enzyme have an easy access to the cell wall (Azad *et al*., 2006).The concentration of cellulase and pectinase were tried for the isolation of protoplast in the present investigation. Among these, combination of cellulase and pectinase (0.1 +0.1 %) showed better response compared with others. The combination of cellulase and pectinase in isolation of protoplast was reported by Ekrem gruel *et al.,* 2002. During protoplast isolation osmotic streangth of cytoplasm and the isolation medium needs to be balanced to prevent plasmolysis or bursting of protoplast. To overcome this 13 % Mannitol was added with enzyme mixture in the present study. The effect of mannitol in plasmolysis was reported by Aziz *et al*., 2006. After purification the isolated protoplast were subjected to fusion. PEG and $CaCl_2$ at high pH were tried for the fusion. Among these the PEG mediated protoplasts fusion was found to be good in the present investigation. Same result was reported by Beranek *et al*. (2007). The protoplast culture meida is often modified from original formulations to suit the requirements of protoplasts from specific species Davey *et al*., 2000. The choice of growth regulator is always critical for successful protoplast culture.Aziz *et al*.,2006 reported that the protoplast of *centella* requires either an ammonium free medium or a low ammonium ion containing medium. B5 medium contains a lowered ammonium ion concentration (Gamborg *et al.,* 1968). So B5 Macronurients and the MS micronutrients were utilized as the basal medium in the present investigation. Same kind of result was also reported in *Ginkgo biloba* (Laurain *et al.,* 1993) and *Daucus carota* (Grambowh *et al*., 1972).

The present study revealed that BA and IAA or NAA are the most effective combinations of plant growth regulators for the callus formation (Table 3) the effect of combination of BA and IAA was reported reported by Takahata and Jomori, 1989 in *Gentiana scabra*; Tremouillaux Guiller *et al*., 1996 in *Ginkgo biloba*, Merchant *et al*., 1997 in *Rose hybrida* and Azad *et al*., 2006 in *Phellodendron amurense rups.*

The fused callus extract and the parental plant extract were subjected to TLC analysis and anti microbial studies for the comparative evaluation. The TLC is very simple, rapid and economical method, and it can be used for the separation of different pigments from the plants (Simona Ioana Vicas *et al*., 2008). The hybridized callus showed higher Rf value compared with parental plants (Table 4, Fig. 6).

The inhibitory effect of medicinal plants on microorganisms due to the presence of the phytochemical components (Babay *et al*., 2004). The result showed the crude extracts of hybridized callus which had higher antibacterial activity compared with parental plant extracts (Table 5, Fig. 5). However, the present study revealed that the hybridized callus had the ability of effective production of secondary compounds than their parental plants.

Conclusion:

Nowadays medicinal plants are widely used for therapeutic purposes by considering their safety and efficacy. Somatic hybridization provides the possibility of producing novel hybrids, with a synergistic combination of secondary metabolites. This work is the first successful attempt for improvement of secondary metabolites by somatic hybridization of *Murraya koenigi* and *Centella asiatica.*

Acknowledgement:

We are greatful to Correspondent and Principal of Malankara Catholic College, Mariagiri for their constent encouragement and support.

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