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Identification of Fungal Contaminant And Standardization of Decontamination Protocol For Micropropagation In *Plectranthus vettiveroides* : A Rich Source of Herbal Medicines

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Abstract: *Plectranthus vettiveroides (K. C. Jacob) N. P. Singh & B. D. Sharma has been used for the preparation of several Ayurvedic medicines as single or as an ingredients but its availability is scanty. The plant is endemic to south India and extinct in the wild and its cultivation requires special agroclimatic conditions. Application of biotechnological intervention is the best option to large scale production of raw material, its conservation as well as genetic improvement. To achieve this goal in vitro multiplication of this crop was tried and found that systemic fungal contamination was a major obstacle. The fungal species invaded on the explant tissues was isolated and its pure cultures established on Potato Dextrose Agar medium. The mycelial and conidial characters were analysed and identified the species as Colletotrichum gloeosporioides. The identity was further confirmed through molecular characterization and standardized remedy to overcome from this fungal attack in in vitro cultures of P. vettiveroides.*

Keywords: *Fungi, Plectranthus vettiveroides, DNA, Colletotrichum gloeosporioides*

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I. Introduction

Plectranthus vettiveroides (K. C. Jacob) N. P. Singh & B. D. Sharma belongs to the family Lamiaceae is an endemic to south Indian aromatic fibrous root yielding herbaceous medicinal plant, which grow well only in moist sandy soil under open sunlight. The essential oil present in the roots of this plant has been used for the preparation of more than 70 different Ayurvedic formulations [1]. But this plant is extinct in the wild and currently cultivated only by few farmers in its original habitat at Kollidam in Cuddalor district, Tamil Nadu, where its production is meager when compared to its demand [2]. In this circumstance, the drug manufactures are forced to use substitutes or adulterants for drug preparation [1]. The plant is propagated through stem cuttings and its floral characters are yet to be described. The stem cuttings brought from Kollidam were established at Saraswathy Thangavelu Centre of JNTBGRI in 2006 and even after eleven years, the crop is not flowered. Considering its medicinal properties, phenology, demand in the pharmaceutical and cosmetic industry, agroclimatic specificity and phytochemical characteristic features, its conservation and development of sustainable utilization strategy through biotechnological intervention is become the need of the hour. Establishment of aseptic cultures under *in vitro* condition is the primary requirement for biotechnological intervention. Selection of explant and its surface sterilization is the *prima face* step for establishing *in vitro* cultures, since nutrient rich medium is supplied for *in vitro* proliferation. Generally, the selected plant parts will be washed with detergent and subsequently treated with single or more than one surface sterlant(s) at different time intervals under aseptic environment. However, in some cases it is very difficult to establish aseptic cultures due to the presence of specific microorganism or special features of mother plant and in such cases identification of the microbial contaminant and standardization of specific treatment system is inevitable. The authors had tried to initiate *in vitro* cultures of *Plectranthus vettiveroides* using the explants such as shoot tip, nodal and leaf excised from field grown plants and observed that fungal contamination was a major obstacle for initiating *in vitro* cultures. In this circumstance, the present investigation was aimed to identify the systemic fungal contaminant associated with the explants and find out proper remedy for successful initiation of shoot cultures.

II. Materials And Methods

Plectranthus vettiveroides germplasm brought from Kollidam in Cuddalor district, Tamil Nadu has been established and maintained at Saraswathy Thangavelu Centre of Jawaharlal Nehru Tropical Botanic

Garden and Research Institute, Puthenthope, Thiruvananthapuram campus through vegetative propagation using top shoot cuttings at 3-4 months interval was selected as the mother plant for *in vitro* culture initiation. Top shoot cuttings having 5-6 nodes (10-15 cm length) were collected from 60-70 days old field grown plants and kept under running tap water for 10-15 min, then immersed in1% (w/w) liquid vim (Hindustan Unilever Limited) for 4-5 min and washed well twice in distilled water. They were brought into laminar air flow hood, then defoliated and the shoot tip and nodal segments having 2-3 cm length were excised and decontaminated using different surface sterilization agents such as 70% ethanol for 30-120 seconds, 5% (v/v) steriliq (Combii Organochem, Pvt. Ltd., New Delhi) for 4-20 min and 0.1% and 0.05% (w/v) $HgCl_2$ for 5-7 min followed by 4-5 rinses in sterile distilled water. To remove the damaged tissues from the cut ends of the shoot tip and nodal explants those tissues were sliced out from the explants using a sharp sterile blade and then they were transferred into both liquid and agar gelled half and full strength Murashige and Skoog (MS) [3] medium supplemented with 3% (w/v) sucrose and 0.6% (w/v) agar. The medium pH was adjusted to 5.8 before autoclaving at 121^{o}C and 1.2 kg cm^{-2} pressure for 18 min. The cultures were incubated at 25 ± 2 ^{o}C under 12 h photoperiod (50-60 mol m^{-2}s^{-2}).

In order to overcome from systemic fungal contamination the most frequently occurred fungal contaminant was isolated and established its pure culture in Potato Dextrose Agar (PDA) medium. The isolate was purified by transferring into fresh culture medium several times. The morphological features of the fungus such as colony description and microscopic characters were documented after 4 -7 days of inoculation.

The isolated fungus was identified using lactophenol cotton blue staining technique [4]. One drop of lactophenol cotton blue stain was placed on a clean microscopic slide and a small portion of fungal mycelium from the cultures was placed in a drop of lactophenol stain. The mycelium was spread very well and covered with a cover slip. The slide was then observed under x10, x40 and x100 magnification lenses respectively and documented its microscopic characters.

Taxonomic identification by molecular characterization and sequence analysis: The fungal mycelia were transferred into 250ml Erlenmeyer's flasks containing potato-dextrose broth without shaking. After 5 days of growth at 28 ± 2◦C, approximately 100 mg of the mycelial biomass was harvested and it was used for molecular characterization.

DNA Isolation: DNA was extracted by modified CTAB method described by Möller *et al*. [5]. The mycelia (50 mg) was scraped from 10 days old fungal cultures and grind manually in 1.5 ml micro-centrifuge tubes with a micro-pestle adding 500 µl of pre-warmed (60°C) TES lysis buffer (100 mM Tris pH 8.0; 10 mM EDTA; pH 8.0; 2% SDS). Then added 50 µg of proteinase K and incubated at 60°C for 60 min. To the suspension 140 µl of 5 M NaCl and 64 µl of 10% (w/v) CTAB were added and incubated at 65°C for 10 min. DNA was extracted by adding equal amount of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 14000 rpm for 10 min. The supernatant was collected and equal amount of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 14000 rpm for 10 min. The DNA was precipitated by adding 0.6 volume of cold isopropanol and 0.1 volume of 3 M sodium acetate, pH 5.2 and maintained at ˉ20°C overnight. The DNA was pelleted out by centrifugation at 12000 rpm for 10 min at 4°C and washed twice with 70% ethanol and suspended in 50 µl TE buffer. RNA was digested by adding 10 mg/ml of RNase and incubated at 37 °C for 45 min and stored at -20 °C.

PCR Amplification of ITS Region: PCR amplification was carried out in 25 µl reaction mixture containing 2.5 µl of 10X amplification buffer (100 mM Tris HCl, pH-8 at 25 °C, 15 mM $MgCl_2$, 500 mM KCl and 10% Triton X-100), 0.2 µl of 25 mM dNTP mixture, 0.74 U of Taq polymerase (Finnzyme, Finland), 1µl each of the primer pair ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGT AAC-3') (Integrated DNA Technologies, Inc., USA) and 40 ng of genomic DNA. Bio-rad thermal cycler (S 1000TM) was used for amplification with the following PCR profile: an initial denaturation for 5 min at 97 °C, followed by 40 cycles of 1 min at 97 °C, 1 min at 48°C and 2 min at 72°C and a final extension at 72°C for 5 min. The amplified products were resolved in 1.2% agarose gel containing 0.5 mg/ml ethidium bromide.

The amplified products were analyzed by agarose gels electrophoresis. The DNA was sequenced using the Applied Biosystems 3730 DNA Analyser (PE Applied Biosystems). The sequence was compared with available data from GenBank databases (http://www.ncbi.nlm.nih.gov/) using the Blastn program. Taxa which showed 99% ITS sequence similarity were noted as reference taxa species and taxa that had less than 99% but more than 95% ITS sequence similarity were identified at the genus level [5]. The closely related sequences obtained through Blastn search were subjected to multiple sequence alignment using the tool Clustal Omega.

In order to eliminate the fungal contaminant from the explants they were first treated with different fungicide such as Blitox^{R} (50% Copper oxychloride), Sultaf^{R} (80% Sulfur) Turf^{R}, Hilzim^{R} (50% Carbendazim) and Dithene M45^{R} for a period of 15 min and then treated with the sodium-hypochlorite and mercuric chloride solutions respectively at different time intervals.

Culture initiation: The surface sterilized shoot tips, node, inter node and leaf segments were cultured in half and full strength MS, media supplemented with 3% (w/v) sucrose, 0.6% (w/v) agar and different individual and permutative combinations and concentrations of 6-benzylaminopurine (BAP), kinetin and α-naphthalene acetic acid (NAA). After one month of inoculation the shoots initiated from the nodal and shoot tips were sub-cultured into fresh medium for multiple shoot initiation and its elongation. Then each shoot 2-5 cm length were separated out and transferred for rooting. The rooted plants were established at 95% success in the field after acclimatization for a period of two weeks in a mist chamber.

III. Results And Discussion

The explants such as shoot tips, node, inter node and leaf segments were surface sterilized following standard method such as cleaning the plant parts gently using detergents followed by successive treatments with more than one surface sterilants at different time intervals such as 70% ethanol for 30-120 seconds, 5-10% sodium hypochlorite for 5-8 min and 0.1% (w/v) $HgCl_2$ for 3-8 min, did not reduce fungal contamination. It was also noted that ethanol treatment induced 100% lethality and sodium hypochlorite above 5% concentration caused bleaching of the tender tissue and tissue death. Use of 0.1% $HgCl_2$ over 5 min also induce tissue death. The standard surface sterilization procedure applied in other *Plectranthus* and *Coleus* species [7,8,9] was found not applicable here. The entire plant body of *P. vettiveroides* has tiny hairs and the removal of fungal spores entangled on the surface of the plant was very difficult. Treatment with fungicide along with detergent and subsequent treatment with 0.1% $HgCl_2$ for 3-5 min could save 50% explants from contamination but it was observed that fungal contamination was emerged from the explant tissue within two weeks of inoculation (Figure 1a). In this circumstance, the fungal contaminant was isolated and established its pure culture through repeated sub-culturing on potato dextrose agar (PDA) medium. The whitish mycilial growth pattern, conidial and other microscopic characteristic features (Fig. 1 b & c) were documented following standard mycological keys and it was identified as *Colletotrichum gloeosporioides*. In order to confirm its identity the DNA was isolated and amplified using the primers ITS4 and ITS5 and sequenced. The obtained nucleotide sequence was as follows.

>TBG New
ACTATGAATAACGCTCTACAACCCTTTGTGAACATACCTATAACTGTTGCTTCGGCGGGT
AGGGTCTCCGCGACCCTCCCGGCCTCCCGCCTCCGGGCGGGTCGGCGCCCGCCGGAGGAT
AACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTT
TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGG
CCCTACAGCTGATGTAGGCCCTCAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCG
TAGTAACTTTACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAATTT
TCCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGC
GGAGGAA

The BLAST similarity analysis using GenBank databases showed the sequence 100% similarity with the species *C. gloeosporioides*. The phylogenetic analysis using Clustal omega showed close relationship with the above species (Fig. 2).

Colletotrichum gloeosporioides is an endophytic fungus; its character may change from intracellular hemibiotrophy to subcuticular nectrophy. So as followed by others [10] the explants were treated with various fungicides at different time intervals. Among the different fungicides used, 80% infection free cultures with less lethality obtained when the explants were treated with Hilzim which contains 50% carbendazim. Chemically carbendazim is methyl benzimidazol-2-ylcarbamate which can inhibit fungal growth [11]. The inhibitory effect of carbendazim on the growth of *C. gloeosporioides* was reported by many authors [12]. The individual and combined use of surface sterilant(s) and the response of explants were documented (Table 1). The surface sterilized explants following the forgoing standardized method produced multiple shoots through the standardization of nutrient and culture condition (Fig 1d.).

IV. Figures And Table

Figure 1. Morphological features of the fungal contaminants observed in *Plectranthus vettiveroides* in *in vitro* culture. (a) fungal growth on the explant, (b) fungal pure culture in PDA medium, (c) fungal condiophore, (d) shoot proliferation from nodal segment inoculated in MS medium supplemented with 3% sucrose .

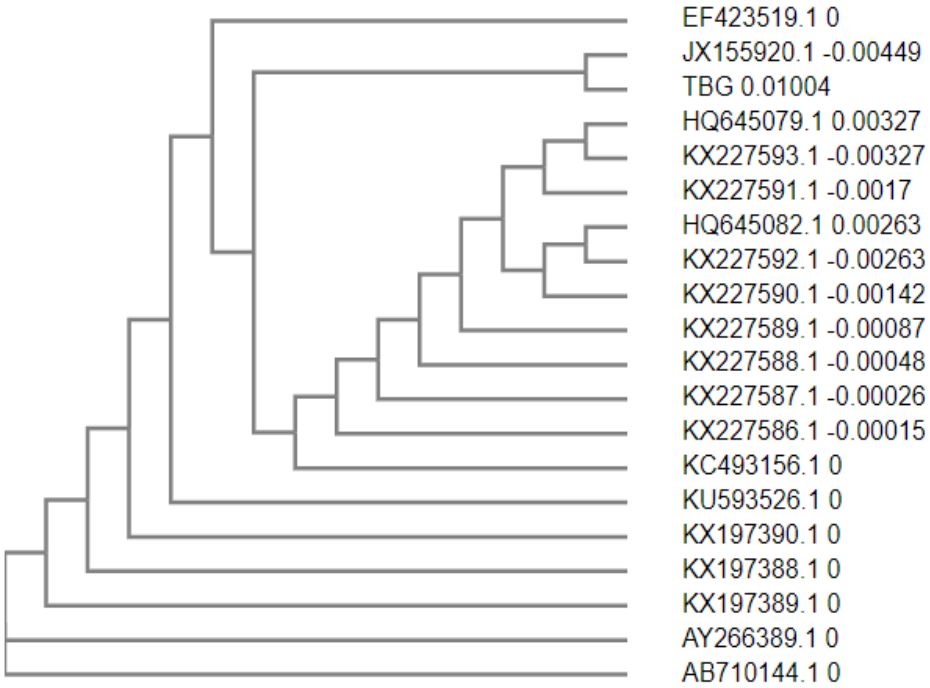

Figure 2. The phylogenetic tree obtained in Clustal omega

Table 1. Survival percentage of explants treated with combination of different fungicide and detergent and subsequent treatments in NaOCl and $HgCl_2$ after 20 days of culture in MS medium supplemented with 3% sucrose and 0.6% agar.

Treatment (10-15 min)	NaOCl (min)	$HgCl_2$ (min)	Survival Percentage
Blitox ^{R} + Vim	3	3	0
Dithene M_{25}+ Vim	3	3	0
Turf ^{R} + Vim	3	3	0
Sultaf ^{R} + Vim	3	3	0
Hilzim ^{R} + Vim	3	3	0
Blitox ^{R} + Vim	4	4	0
Dithene M_{25}+ Vim	4	4	0
Turf ^{R} + Vim	4	4	0
Sultaf ^{R} + Vim	4	4	0
Hilzim ^{R} + Vim	4	4	0
Blitox ^{R} + Vim	5	5	0
Dithene M_{25}+ Vim	5	5	0
Turf ^{R} + Vim	5	5	0
Sultaf ^{R} + Vim	5	5	0
Hilzim ^{R} + Vim	5	5	0
Blitox ^{R} + Vim	4	3	0
Dithene M_{25}+ Vim	4	3	0
Sultaf ^{R} + Vim	4	3	0
Hilzim ^{R} + Vim	4	3	0
Blitox ^{R} + Vim	-	3	0
Hilzim ^{R} + Vim	**-**	**3**	**80**
Blitox ^{R} + Vim	-	4	0
Hilzim ^{R} + Vim	**-**	**4**	**60**
Blitox ^{R} + Vim	-	3	0
Hilzim ^{R} + Vim	-	3	0
Hilzim ^{R} + Vim	8	-	0
Hilzim ^{R} + Vim	5	3	0

V. Conclusion

Establishment of microbial free culture system is the pre-requisite for the micropropagation of any crops and in the present study it was achieved through identification of the microbial contaminant persisting even after applying general decontamination procedure and also proposed appropriate remedies for establishing contamination free *in vitro* cultures of *Plectranthus vettiveroides*.

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