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Research Article

IDENTIFICATION AND BIOASSAY OF FUNGAL CONTAMINANTS OBSERVED DURING *IN VITRO* PROPAGATION OF *Saraca asoca* (Roxb.)DE WILDE

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ABSTRACT

Attempts were made to establish contamination free cultures of *Saraca asoca* through bioassay and evaluation of suitable explants and medium for micro-propagation of *S. asoca.* During the experiment, fungal contaminants were observed on explants even after complete sterilization. The pure cultures prepared along with contaminants were identified according to their morphological and cultural characteristics. The identified systemic fungus comprised of *Fusarium lateritium* and *Colletotrichum gloeosporoides* contaminating the *in vitro* cultures of *Saraca asoca* after 2 to 3 weeks. Bioassays were established using three different concentration of fungicides viz. Sectin, Bayleton, Indofil, Bavistin, Copper oxychloride, Propiconazole. The fungicide Propiconazole was found to completely inhibit the growth of the fungal isolates at 0.05% premixed in PDA medium. The pre-sterilization treatment of explants of *S.asoca* with Propiconazole revealed that maximum contamination free cultures (86.7%) were obtained on the Nistch Medium supplemented with 0.5 BAP, 0.5mg/l plant growth hormone, and 0.25mg/l defol using control.

Key words: *Saraca asoca*, *in vitro* culture, fungicide, bioassay, Propiconazole, Nistch medium.

INTRODUCTION

Ashoka, *Saraca asoca* (Roxb.) De Wilde (family Caesalpinaceae) is one of the important medicinal species known for its variety of pharmacological activities. The species is listed as one of the 32 priority species by National Medicinal Plant Board (NMPB). The species is vulnerable (Taylor, 2000) and is at the verge of extinction due to unscientific harvesting of bark and other plant parts for medicinal uses. The ever

increasing demand of this species and high marketability calls for development of technologies which could be utilized for mass production. Tissue culture is a proven technology for mass multiplication and conservation of many threatened woody species.

Often it is difficult to initiate and maintain cultures of mature woody species due to infestation of systemic fungi. A wide range of microorganisms (filamentous fungi, yeasts, bacteria, viruses and viroids) and micro-arthropods (mites and thrips) have been identified as contaminants in plant tissue cultures. Contaminants may be introduced with the explant, during manipulations in the laboratory, by micro-arthropod vectors (Tanprasert and Reed, 1997; Leifert and Cassells, 2001) or endophytic bacteria (Reed *et al.,* 1995; Pereira *et al.,* 2003). Fungus may arrive with an explants, or airborne, or enter a culture (Babaoglu *et al.,* 2001). Frequently encountered bacterial and fungal contaminations especially in laboratories of commercial micro propagation pose a considerable problem (Reed *et al.,* 1998). Studies on the effect of antibiotics and fungicides on these kinds of contaminantswere carried out by George (1993).

Present experiment was framed to identify fungi contaminating cultures and establish bioassays to eradicate the contamination in order to obtain contamination free cultures of *S. asoca* through various fungicides and evaluation of suitable explants.

MATERIAL AND METHODS

Explants of *Saraca asoca* were collected from trees (below 10 years age) growing in Forest Research Institute, Dehradun. Collected shoots were kept in plastic cover. After excision of leaves, shoots were cut into 2.5-3.0 cm long apical and nodal shoot segments. To remove dust particles from the explants surface, explants were dipped in Tween-20 (0.1%, v/v) liquid detergent solution for 15 minutes, shaked periodically and washed 3-4 times with double distilled water to remove carryover effect of detergent. In order to minimize fungal contamination, explants were treated with 0.1% (w/v) solution of Bavistin (Carbendazim 50% WP- a systemic fungicide) for 15 min and washed 3-4 times with double distilled water. The treatment of 0.5% streptomycin was given to explants to remove bacterial contaminants. Later on, explants were surface sterilized with 70% (v/v) ethanol for 50 seconds and washed 3-4 times with sterile double distilled water. The two sterilizing agents' viz. mercuric chloride and sodium hypochlorite were used for final sterilization of explants in laminar airflow.

The contaminated cultures were used to prepare pure cultures for fungal identification through microscopic examinations. Then, the bioassay experiment was carried out to check the inhibition of identified fungi against different concentrations of tested fungicides. In this study, three different concentrations (0.05 pp, 0.1 pp, 0.15 pp) of six fungicides viz. Sectin, Bayleton, Indofil, Bavistin, Copper oxychloride, and Propiconazole were used. The observations were recorded on the basis of growth (diameter in cm.) of

fungal colony on fungicides supplemented PDA. Further the rate of inhibition was calculated by using following formula. The effective fungicide was incorporated in the pre-treatment protocol for sterilization of explants of *S. asoca.*

$$\% \text{ inhibition} = (T - C) / C * 100$$

T = Diameter of fungal colony on fungicide supplemented medium, C = Diameter of fungal colony on control.

RESULTS AND DISCUSSION:

In vitro protocol for *S. asoca* was standardized at Nistch Medium supplemented with 0.5 BAP, 0.5 mg/l plant growth hormone, and 0.25 mg/l Defol (Himedia) in whole experiment for maximum sprouting response. Fungal characterization and bioassay were required for the sake of establishment of aseptic cultures of *S. asoca*. Maximum percentage of aseptic cultures was obtained at 0.2% $HgCl_2$ followed by 0.15% $HgCl_2$ treatment. But the maximum survival % was observed at 0.15% $HgCl_2$ treatment for 15 minutes (Table. 1).

Table. 1. *In vitro* $HgCl_2$ sterilization treatment for three different time intervals

	TIME					
	5 minutes		10 minutes		15 minutes	
Conc.	Aseptic cultures (%)	Survival %	Aseptic cultures (%)	Survival %	Aseptic cultures (%)	Survival %
0.10%	0	0	4	0	32	20
0.15%	40	32	84	68	88	72
0.20%	88	40	92	24	92	8

Significance level at 0.05%

Aseptic culture			
LSD	conc.	0.1089	Significant
	Time	0.1089	Significant
	Conc. * time	0.1886	Significant

f-value	conc.	<0.001
	Time	<0.001
	Conc. * time	0.001

Most of the *in vitro* cultures were contaminated after few weeks due to systemic infection even after complete sterilization with $HgCl_2$- Bavistin (Figure.1). The pure cultures prepared from the

contaminated cultures showed white and brown colored fungal colonies (Figure.2).

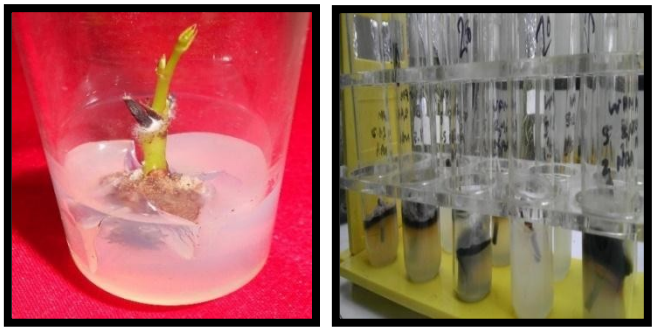

Figure.1. Cultures contaminated with *Fusarium lateritium* after 2-3 weeks.

Figure. 2. Pure cultures prepare for two systemic funguses on PDA.

Microscopic and morphological identification of pure cultures showed that two fungi *Fusarium lateritium* and *Colletotrichum gloeosporoides* were found to contaminate the *in vitro* cultures of *S.asoca* after 2 to 3 weeks. Mostly, *Fusarium lateritium*a white cottony slow growing systemic fungus was found on explants inhibiting the growth of *S.asoca* under *in vitro* condition. Reed *et al.* (1998) also showed internal bacterial contamination in hazelnut shoot cultures and contaminants evident at culture establishment, or became apparent after several subcultures.

Bioassay experiment showed that out of six fungicides used only Propiconazole completely inhibited the growth of the fungus at all three concentrations used (Figure. 3 and 4). Burun *et al.,* (2010) also identified contaminants *in vitro* culture of *Lilium candidum* according to their morphological and cultural characteristics comprising of *Fusarium, Penicillium, Alternaria, Rhizopus, Cylindrocarpon* and *Aspergillus* species. The most effective treatment against fungal contaminations was achieved by utilizing a combination of Benomyl and Nystatin.

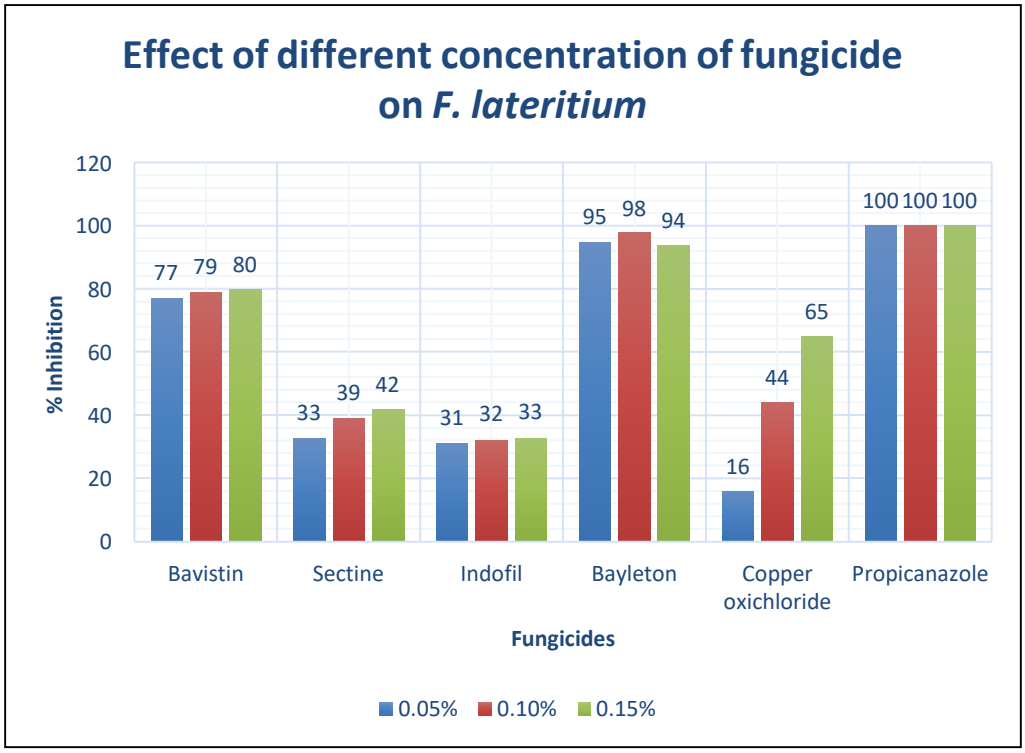

Figure. 3. Effect of different concentration of fungicide on *F. lateritium*.

(i)Control **(ii) 0.05 %** **(iii) 0.1 %** **(iv) 0.15 %**

Figure.4. Growth inhibition of *F. lateritium* using Propiconazole supplemented PDA medium using control.

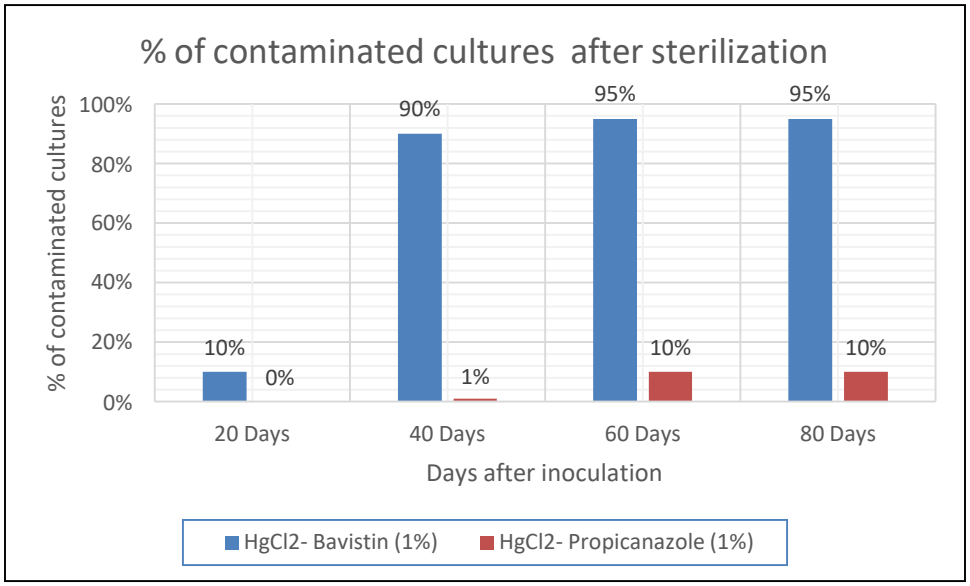

Figure. 5. Percentage of contaminated cultures after sterilization

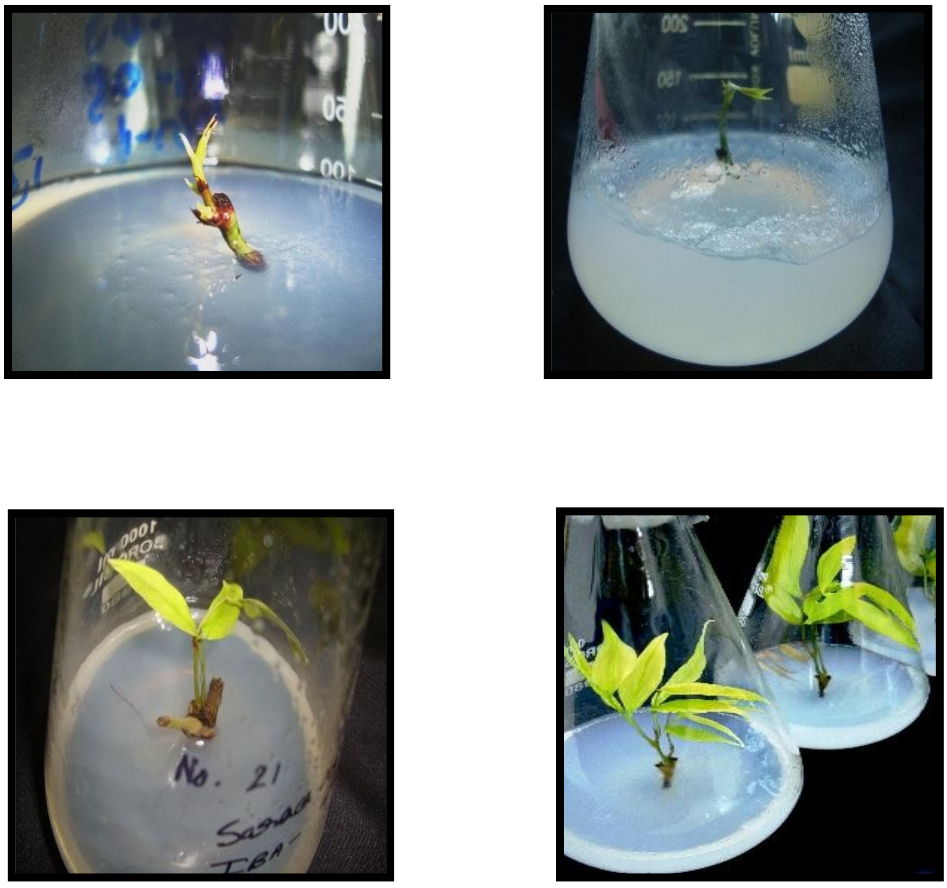

Figure.6.*In vitro* cultures after sterilization of explants with Propicanazole

Comparison with $HgCl_2$- Bavistin (1%) mediated sterilization of explants with $HgCl_2$- Propicanazole (1%) for 15 minutes showed only 10 % contaminated cultures after 60 days. Whereas in $HgCl_2$– Bavistin protocol survived culture invariably become contaminated (95%) with systemic fungus *F. lateritium* after 60 days (Figure. 5). A successful bud break and leaf formation was observed after sterilization with Propiconazole (Figure. 6).

CONCLUSION

The outcome of the work would lead to development of an efficient protocol for multiplication of this recalcitrant and medicinally important species which otherwise is difficult to propagate due to unavailability and poor viability of seeds. The effective fungicide (Propicanazole) was incorporated in the protocol for sterilization experiment of explants of *S. asoca.* It was found highly effective to act on systemic fungus *Fusarium lateritium* which was previously contaminating the *in vitro* cultures.

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