Journal of Medicinal Plants Research, Vol. 4(4) pp. 286-292, 18 February, 2010
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875© 2010 Academic Journals

Full Length Research Paper

A survey on VAM association in three different species of *Cassia* and determination of antimicrobial property of these phytoextracts

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Accepted 9 November, 2009

Demands of traditional herbal medicines are increasing day by day over the world. Considering the growing demand of medicinal plants in curative treatments to our society and the role of VAM fungi in augmentation of the production of active secondary metabolites by the medicinal plants, the present work has been undertaken to survey the mycorrhizal status in three different species of *Cassia viz, C. alata, C. occidentalis and C. sophera* having ethnobotanical uses. From Grid-line intersect method it was observed that *C. alata* possesses maximum root colonization (68 - 71%) by the VAM fungus followed by *C. occidentalis* (45 - 57%) and *C. sophera* (24 - 30%). Spore analysis from the rhizosphere soil sample exhibited a great deal of variation in their morphological features and they mostly belong to the species group of *Glomus*. An attempt has been made to study the antimicrobial property of the leaf extract of all of these three species of *Cassia* against a few gram positive and gram negative bacteria as well as against a few fungi which are mostly dermatophytes causing skin infection in human beings. It seems that *C. alata* is the most potent species for having significant antimicrobial activity.

Key words: *Cassia alata*, *Cassia sophera*, *Cassia occidentalis*, VAM, antimicrobial property.

INTRODUCTION

The symbiotic association between fungi and the roots of higher plants comes under the general name 'mycorrhiza' which literally means fungus- roots. Vesicular arbuscular mycorrhizal fungi (VAM) or most commonly arbuscular mycorrhizal fungi (AM) are ubiquitous in their distribution and occur abundantly (Gabor, 1991; Power and Bagyeraj, 1986; Harley and Smith, 1983). Majority of flowering plants have dynamic association of VAM fungi. The significance of VAM in augmenting food production is increasingly appreciated. VAM fungi are responsible for enhanced uptake of mineral nutrients especially phosphorus from the soil by the host plants and thereby enhancing vigor (Gerdemann, 1964; Gerdemann, 1971; Nicolson, 1967; Mosse, 1972; Barea et al., 1988). That is why they are referred as 'biofertilizers' and can be substantiated for the substantial amounts of chemical fertilizers. Despite the importance of mycorrhizae in agriculture and forestry, little work has been done regarding their distribution, diversity and association with the host plants in India.

Traditional herbal medicines are increasingly being used not only by the developing countries but also by the developed countries in their primary health care system. A bulk of our rural population relies on the drug resources of plant origin. Thus, the cultivation of medicinal plants is increasing steadily to maintain a steady supply and to

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support their increasing demand. But corresponding research works on the occurrence of VAM fungi and their associations in medicinal plants have received very little attention as compared to the studies on forest species and field crops. Thus, the purpose of present study is to investigate the extent of VAM association in some common medicinal plants. In present communication three different species of *Cassia viz, C. alata, C occidentalis and C. sophera* were taken into consideration. All of these three different species of *Cassia* have got their tremendous medicinal importance, antidiabetic, anti-inflamatory, anticonvulsant, antioxidant, antifungal and antibacterial properties.

The active compounds isolated from *C. alata* contain some flavonoid compounds like anthroquinones sennosides, kaempferol etc. which are used in the treatment of constipation, fistula, inguinal hernia, intestinal parasitosis, blennorrhagia, syphilis and diabetes (Abo et al., 1998; Adjanahoun et al., 1991; Kochar, 1981). *C. alata* is commonly known as 'dadmari' which is extensively used against fungal dermatophytes and other skin infections. Present communication is dealt with an investigation on VAM association in these three different species of *Cassia and* determination of antimicrobial property of these phyto-exrtacts. During surveys, soil types, soil nutrient levels like nitrogen and phosphorus content of the soil and pH of the soil were taken into consideration to determine the effects of these soil parameters on root colonization by VAM fungi. Seasonal variations were also taken into account. Antimicrobial properties of the phytoextracts were determined against some pathogenic fungi and bacteria.

MATERIALS AND METHODS

Site of collection

The sites chosen for collection were Ramnabagan forest and Golapbag of The Burdwan University campus and adjoining areas of Burdwan district and thorough survey was conducted for collection of root samples from three different species of *Cassia*.

Identification of the plants

The plants were identified by using the specimens of herbarium, Botany Department, Burdwan University, Burdwan.

Collection of root samples

For each species, the feeder roots were collected directly from the plants by digging and tearing the roots up to the base of the main stem.

Maintenance and preservation of roots

The root samples after collection were thoroughly washed in running tap water and rootlets were selected and cut into small pieces and fixed in formaldehyde/acetic acid solution (Johansen, 1940).

Collection of soil sample

Soil sample of about 10 gm was collected from the root region (rhizosphere) of each of the three species of *Cassia* by digging the soil up to a depth of 10 cm and collected into polythene bags, labeled and stored at 4ºC until analysis.

Preparation of root samples

For each specimen, 100 feeder root pieces were thoroughly washed in water and boiled at 95ºC temp. for different durations (like 10, 15, 20, 25 and 30 min) in 10% KOH. The segments were washed in distilled water, acidified with 1(N) HCL and stained in 0.05% trypan blue in lactophenol, the excess stain was removed by washing with lactophenol. Root segments were mounted temporarily on slides in acetic acid; glycerol (1:1 V/V) and the edges of the cover slips were sealed with DPX and observed under microscope (Leica, model no. DMLB).

Assessment of vesicular arbuscular mycorrhizal (VAM) fungal association in roots

The vesicular arbuscular mycorrhizal (VAM) association in each specimen was examined in the roots following the method of Phillips and Haymann (1970) and was calculated as percentage of mycorrhizal association.

$$\% \text{ Mycorrhizal association} = \frac{\text{No. of mycorrhiza associated segments}}{\text{Total no. of segments scored}} \times 100$$

Collection of mycorrhizal spores from soil samples

At first 10 gm soil sample was taken and dissolved in 100 ml distilled water in a conical flask. Then the conical flask was shaken for 30 min and after that the conical flask was kept in undisturbed condition for 30 min. So that the soil particles can precipitate and the spores were floated on the surface of the liquid. Mycorrhizal spores were obtained by wet sieving and decanting (Gerdemann and Nicolson, 1963). The solution was passed through 250, 150, 53 and 45 µm sieve and the spores were collected from the residue present on the sieves. Spores of large size were considered. This residue was dissolved in distilled water and filtered. The residue present in the filter paper was taken and mounted on a slide in lactophenol and cotton blue and were examined under microscope (Leica, model no. DMLB).

Spore count

VAM fungal spores were extracted from three replicates of 50 g soil by wet sieving and decanting technique (Gerdemann and Nicolson, 1963). The decantant were filtered through a filter paper with grid lines. The filter paper was then spread on a glass plate under a dissecting microscope and counted and expressed as spores per 10 g of dry soil.

Study of the antimicrobial activity of the phytoextracts

Antifungal activity

In vitro effect of the phytoextracts (ethanolic extract of leaf) of *C. sophera, C. alata and C. occidentalis* against some common fungal and bacterial strains was studied. Antifungal activity of the phytoextracts was studied following cup- plate assay (Royse and Ries, 1978) and food poisoning technique (Mondal et al., 1995).

Cup plate assay

In cup plate method, 15 ml of sterilized PDA medium at pH 6.5 was plated in each of the Petri dishes and allowed to solidify. A groove was made in the centre of the PDA plate with the help of a cork borer in which 3 ml of each of the phytoextracts were added. Five mm inoculum disc cut from the actively growing culture of each of the test fungal strains was placed on one side of the medium in the same plate and was incubated at 30 ± 1ºC for 7 days. At the end of the incubation period, the radial growth of the test fungal strain was measured separately and the percentage of growth inhibition of the pathogen was recorded. Five replica plates were made for each treatment.

Food poisoning assay

For designing the 'food poisoning technique', 15 ml of PDA medium was poured in each of the sterilized Petri dishes and allowed to solidify. Just before solidification 3 ml of each of the phytoextracts were added to the Petri dishes separately and mixed thoroughly, inoculated with mycelial discs (5 mm) of each of the test fungi at the centre of the Petri dishes and incubated at 30 ± 1ºC for 7 days. At the end of incubation period, the diametric growth of the test fungi were recorded and percentage growth inhibition of the fungi were calculated against the control set. Five replicates were made for each treatment.

Antibacterial activity

Antibacterial effects of the phytoextract of *cassia alata* were studied following agar-well diffusion technique (Akpata and Akinrimisi, 1977) with little modification. An overnight culture of each microbial isolates was emulsified with nutrient broth to a turbidity that was equivalent to 0.5 McFarland (10^8 cfu/ml). 100 µl of each standard inoculum was then poured on nutrient agar and PDA at 10^6 cfu/ml per spot to attain a confluent growth for 24 h. at 37 ± 1°C (Bauer et al., 1966). Wells were made on the agar using a sterile cork borer and filled with 100 µl of the respective extract. The plates were incubated. Bacterial and fungal control wells contained 100 µl of ciprofloxacin and fluconazole, respectively, at 5 µg per well. A well containing 100 µl of Tween-20-PBS solution (pH 7.2) was generally used as a positive control well in every assay. Growth inhibition was measured as diameters of inhibitory zone.

Estimation of soil Phosphorus and Nitrogen

Soil of the study site was analysed for pH value and for available phosphorus and nitrogen present in the soil. Soil samples were collected from the rhizosphere zone at a depth of 1 - 2 cm and air-dried and passed through a 2 mm pore size sieve for determining available N and P. Available N was determined by alkaline permanganate method and available P was measured by Olsen reagent (Olsen et al., 1954).

RESULTS AND DISCUSSION

The soil pH was more or less neutral to mild alkaline and soil phosphorus content varied from 0.58 to 0.72 mg/Kg in the rhizosphere. All the three species of *Cassia* showed mycorrhizal colonization and a wide range of variations was exhibited in root colonization percentage among different species which might be due to the effect of rhizosphere soil that favored the growth of AM fungi. AM fungal colonization percentage was higher in *C. alata* (68 - 71%) followed by *C. occidentalis* (45 - 57%) and *C. sophera* (24 - 30%). Spore count per 10 gm of dry soil in the rhizosphere ranges from 19 - 106 and it was recorded to be the maximum in *C. alata* (86 - 106). Spores and vesicles of mycorrhizal fungi in the *cassia* species exhibited a great deal of variations in their morphology. The VAM fungi found here were identified by using standard manual and the synoptic key of Schenck and Perez (1997) and Trappe (1982). It has been noticed that soil phosphorus content influences VAM colonization percentage in a greater extent and an increase in available pool of phosphorus in the soil profoundly decreases the root infection and root colonization percentage by the AM fungi as evidenced in the present study.

Furthermore, a phosphorus deficient condition in the soil favored mycorrhizal colonization in the roots. However, soil pH has no marked effect on root colonization by the AM fungi though in the present study it remained within a very narrow range (6.8 - 7.3). It is conclusive from the results that summer season is the best for efficient growth and root colonization by AM fungi in the field followed by spring. The results are corroborative with the findings of Setua et al., (2001) who observed maximum number of AM spores in Mulberry during summer than winter. It might be due to better symbiosis between the mycorrhizal fungi and the host roots under congenial environment. Enhanced growth of the resident AM fungal flora and root colonization during summer than winter has been reported by several authors like Hayman (1974), Koske (1981), Kabir et al. (1997), Merryweather and Fitter (1998).

It might be due to the fact that higher temperature during summer affects the pre-infection stage of the establishment of mycorrhizal symbiosis and increases the number of entry points in the host root. Higher light intensity in summer may well be a causative factor for enhanced development of infection peg which in turn may lead to the development of an increased number of arbuscules. However, the level of AM fungal association is very much dependent on root morphology, metabolism as well as plant growth (Warner and Mosse, 1980).

Among the phytoextracts of different species of *Cassia* tested, *C. alata* exerted maximum antimicrobial property. It inhibited the radial growth of some common human pathogenic fungi like, *Candida albicans* (83.34%), *Aspergillus candidus* (71.11%), *Aspergillus flavus* (68.89%), *Rhizopus stolonifer* (68.89%) and *Penicillum patulum* (66.67%). The results are corroborative with the findings of Makinde et al. (2007), Souwalak et al. (2004) who observed antimicrobial property of the leaf extract of *C. alata* against some human pathogenic bacteria and fungi. Methanolic extract of *C. alata* showed antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus.* These results are concomitant with the earlier findings of Somchit et al. (2003). Further, antibacterial activity of leaf extract of *C. alata* has been reported by Owoyale et al. (2005) against *Escherichia coli*, *B. subtilis, Salmonella*

Table 1. Incidence and extant of mycotrophy in three different species of *Cassia.*

Name of the plant	Season of collection of the roots	Presence or absence of mycorrhizae	Mycorrhizal infection*		
			Vesicles	Arbuscules	% Root colonization
C. occidentalis	Winter	+	+	+	45± 11
	Spring	+	+	+	57 ± 12
C. alata	Summer	+	+	+	68 ± 11
	Summer	+	+	+	71 ± 13
C. sophera	Spring	+	+	+	24 ± 09
	Summer	+	+	+	30 ± 10

*Data are the mean values of five replicates; + Indicates presence of mycorrhizae

Table 2. Soil nutrient parameters and mycorrhizal colonization in the rhizosphere soil of three different species of *Cassia.*

Name of the plant	Soil type	Soil nutrient / soil nutrient character (mg/Kg soil)			No. of spores /10 gm soil*
		Phosphorus (mg)	Nitrogen (mg)	PH	
C. occidentalis	Open grass land soil	0.66 ± 1.46	88.47 ± 3.35	6.8	48 ± 11
	Forest soil	0.72 ± 2.35	90.56 ± 2.22	7.3	67 ± 6
C. alata	Open grass land soil	0.58 ± 1.78	86.75 ± 4.11	7.1	86 ± 9
	Forest soil	0.72 ± 3.12	91.34 ± 5.25	7.3	106 ±13
C. sophera	Open grass land soil	0.65 ± 1,12	77.21 ± 6.12	6.9	19 ± 7
	Forest soil	0.72 ± 2.95	69.90 ± 2.88	7.3	23 ± 5

*Data are the mean values of five replicates.

Table 3. Antimicrobial effect of three different species of *Cassia* on some selective fungi following 'cup-plate assay'.

Phytoextract	Test fungi	Colony diameter of the fungal Pathogen (c.m.)	% Growth inhibition of the fungal pathogen *
C. alata	*Aspergillus niger*	2.7	70.00±0.04
	Aspergillus flavus	3.2	64.45±0.04
	Aspergillus candidus	2.5	72.23±0.02
	Penicillium patulum	4.0	55.56±0.01
	Candida albicans	2.2	75.00±0.05
	Rhizopus stolonifer	3.0	66.67±0.09
C. occidentalis	*A. niger*	4.5	50.00±0.05
	A. flavus	5.4	40.00±0.01
	A. candidus	8.6	04.44±0.03
	P. patulum	5.6	37.78±0.08
	C. albicans	4.0	55.56±0.05
	R. stolonifer	5.2	42.22±0.09
C. sophera	*A. niger*	7.4	17.78±0.24
	A. flavus	8.0	10.11±0.16
	A. candidus	9.0	00
	P. patulum	6.0	33.33±018
	C. albicans	8.5	5.56 ±0.24
	R. stolonifer	5.6	37.77±0.24

Table 3. Contd.

Fluconazole antibiotic (100 µl at a dose of 5 µg/ml)	*A. niger*	0.5	94.44 ± 1.26
	A. flavus	0.5	94.44 ± 1.10
	A. candidus	1.0	88.89 ± 2.12
	P. patulum	1.7	81.11 ± 0.65
	C. albicans	1.3	85.56 ± 0.83
	R. stolonifer	0	100 ± 0.12
Control		9.0	-

*Data are the mean values of five replicates. CD at 5% → 1.16.

Table 4. Efficacy of the phytoextracts against mycelial growth of the test pathogenic fungi following 'food poisoning technique'.

Phytoextract	Test fungi	Colony diameter of the Pathogen (c.m.)	% growth inhibition of the pathogen *
C. alata	*A. niger*	2.0	77.78 ± 2.31
	A. flavus	2.8	68.89 ± 1.22
	A. candidus	2.6	71.11 ± 2.12
	P. patulum	3.0	66.67 ± 1.89
	C. albicans	1.5	83.34 ± 0.90
	R. stolonifer	2.8	68.89 ± 1.40
C.occidentalis	*A. niger*	3.7	58.88 ± 2.28
	A. flavus	4.3	52.22 ± 2.51
	A. candidus	7.0	22.22 ± 1.10
	P. patulum	5.4	40.00 ± 0.90
	C. albicans	3.6	60.00 ± 1.12
	R. stolonifer	4.4	51.11 ± 0.87
C. sophera	*A. niger*	7.0	22.22 ± 0.75
	A. flavus	7.5	16.67 ± 1.11
	A. candidus	8.0	11.11 ± 0.95
	P. patulum	5.6	37.78 ± 1.23
	C. albicans	7.5	16.66 ± 1.34
	R. stolonifer	4.9	45.56 ± 1.25
Fluconazole antibiotic (100 µl at a dose of 5 µg/ml)	*A. niger*	0.5	94.44 ± 0.70
	A. flavus	0	100 ± 0.10
	A. candidus	0.8	91.11 ± 0.05
	P. patulum	1.5	83.33 ± 0.03
	C. albicans	1.0	88.89 ± 0.44
	R. stolonifer	0	100 ± 0.26
Control		9.0	-

* Data are the mean values of five replicates; CD at 5% → 2.93.

typhi, Pseudomonas aeruginosa and S. aureus and by Makinde et al. (2007) against *Dermatophilus congolensis and Actinomyces bovis*. Crude ethanolic and water extracts of leaves and stem bark of *C. alata* exhibit significant antibacterial property against *S. aureus* (Somchit et al., 2003). Tables 1 - 5.

However, it is imperative from the results that antifungal property of the leaf extract of *C. alata* is more pronounced than its antibacterial activity. Mycorrhizal fungi – a boon for sustainable agriculture and crop productivity.

Table 5. Antibacterial activity of the phytoextracts against some pathogenic bacteria.

Phytoextract	Name of the bacteria	Growth status*
C. alata	*Vibrio cholerae*	+
	Bacillus subtilis	+ + + +
	Staphylococcus aureus	+ + +
	Streptococcus sp.	+ + +
	Escherichia coli	+ +
C. occidentalis	*V. cholerae*	-
	B. subtilis	+ +
	S. aureus	+
	Streptococcus sp.	+
	E. coli	+
C. sophera	*V. cholerae*	-
	B. subtilis	+
	S. aureus	-
	Streptococcus sp.	-
	E. coli	-
Ciprofloxacin antibiotic (100 µl at a dose of µg/ml	*V. cholerae*	+ + + + +
	B. subtilis	+ + + + + +
	S. aureus	+ + + + + +
	Streptococcus sp.	+ + + + + +
	E. coli	+ + + + + +
0.1% (v/v) Tween-20 in phosphate buffer saline (P^H 7.2).	*V. cholerae*	G
	B. subtilis	G
	S. aureus	G
	Streptococcus sp.	G
	E. coli	G

*Data are the mean values of five replicates
CD at 5% → 1.82
+ = 5-10 mm diameter of zone of inhibition, ++ = 10-20 mm diameter of zone of inhibition.
+ + + = 20-30 mm diameter of zone of inhibition, + + + + = 30-40 mm diameter of zone of inhibition.
+ + + + + = 50-70 mm diameter of zone of inhibition, + + + + + + = 70-95 mm diameter of zone of inhibition
G = Growth of bacteria around the Tween-20 control well.

They are also associated with medicinal plants and improved their growth and vigor (Trivedi, 2007). Mycorrhiza inoculated plants showed significant increase in growth and production of active secondary metabolites in some medicinal plants *viz*. *Hemidesmus indicus, Gymnema sylvestris, Andrographis paniculata, A. alaba and Clerodendrum phlomidis* (Sampathkumar, 2007). Antimicrobial property of *C. alata* is mainly due to the presence of high amount of phenolic compounds (Makinde et al., 2007). As VAM association enhances the secondary metabolite production along with enhanced growth of the of medicinal plants (Sampathkumar, 2007), so it is conclusive that association of VAM fungi in test species of *Cassia* may enhance their productivity of crude secondary metabolites and subsequently antimicrobial property.

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