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Isolation and identification of flavonoid from mycorrhizal and non mycorrhizal roots and rhizomes of *Acorus calamus*

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Abstract

Soil samples were collected and were mixed thoroughly and a portion of soil was analysed for soil texture. Mycorrhiza Helper Bacterium (MHB) and Plant Growth Promoting Rhizomicroorganisms (PGPR) have emerged as the important component of integrated plant nutrient supply system and hold a promising source for reducing the cost, to improve the crop yields, quality, nutrient supplies and sustaining the productivity over a long period. Inoculation of mycorrhizal species enhanced the plant growth, rhizome length and diameter, biomass, nutrition as well as root colonization and spore numbers in the root zone soil when compared to uninoculated treatment. Specific flavonoid, kaempferol and its 3-O-rutinoside were isolated from mycorrhizal and non-mycorrhizal roots and rhizomes of *A.calamus*. There was no change in structure of flavonoid viz., kaempferol and quercetin due to inoculation. Flavonoids, phenolic compounds and carbohydrates were present in all the organic solvent extracts in the test plant. The present investigation clearly showed that the inoculation of mycorrhizae proved to have synergistic effects on the growth response, per cent of alkaloid and specific flavonoid as compared to inoculation with mycorrhizae alone.

Keywords: Mycorrhizae, flavonoid, roots, MHB, PGPR, rhizomes and soil texture

INTRODUCTION

Some soil bacteria, which have been named Mycorrhizal Helper Bacteria (MHB), could enhance the development of the mycorrhizal symbiosis (Garbaye, 1994). It is well known that a considerable number of bacterial species are also able to exert a beneficial effect on plant growth. Medicinal plants in India were originally reported to be non-mycorrhizal probably due to the presence of various secondary metabolites (Mohankumar and Mahadevan, 1984). However, roots of field grown garlic were found to be colonized by Arbuscular Mycorrhizal fungi (Shuja and Khan, 1977) and this observation has more recently been supported by many workers from Asia who found the roots of various medicinal plants to be mycorrhizal (Laksman and Rahavendra, 1990).

Presently, considerable importance is being given to AM fungi and MHB, because of awareness of environmental pollution and health hazards by the use of chemicals. The role of AM fungi and MHB in improving plant growth is well documented (Lakhman ,1992; Murthy *et al*., 1998). However, Troppe (1984) for the first time reported the presence of AM fungi in underground storage organs. They reported the presence of arbuscular mycorrhizal association in the vascular system of rhizomatous tissue and the scale like leaves of *Zingiber officinale*. They reviewed the presence of AMF associated with the portion other than roots in twenty one angiosperms and non angiosperms species. Then several papers reported the incidence of AM fungal colonization in underground storage organs of *Acorus calamus* (Selvaraj, 1989).

MATERIALS AND METHODS

Soil samples were collected and mixed thoroughly and a portion of soil was analysed for soil texture, pH, ECsc, OM, N, P, K, Zn, Ca, Mn and Fe at the soil testing laboratory, TamilNadu Rice Research Institute, Aduthurai, Tamil Nadu following standard methods (Piper, 1950; Jackson, 1973 and Sharma *et al*., 1986). Spore population of soil sample was estimated by a modified wet-sieving and decanting technique (Gerdemann and Nicolson, 1963).

Extraction and fractionation

Mycorrhizal and non mycorrhizal fresh rhizomes and roots of *Acorus calamus* were extracted separately with 85% methanol (5×500ml) under reflux. The alcoholic extract was concentrated in vacuum and the aqueous extract was successively fractioned with petroleum ether (60-80°) (4×250ml), peroxide free Et_2O (3×250ml) and EtOAc (4×250ml). The petroleum ether fraction did not yield any crystalline solid.

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Et_2O fraction (Flavonol:Kaemferol)

The Et_2Ofraction was concentrated in vacuum. The residue from Et_2O fraction was taken up in Me_2Co and left in an ice-chest for a week. An yellow solid that separated was filtrated and recrystallized from methanol when pale yellow needles were obtained (m.pt.277-279°C, yield 0.05%). It was readily soluble in alcoholic solvents and sparingly soluble in hot water. It develop a red colour with Mg-HCl and yellow colour with NH_3. It responded to Harhammer-Hansel, Wilson's Boric acid and Gibbs tests, but did not answer Molisch's test. It had theλ_{max} (UV data) values are presented in Table 1. Its Rf values are given in Table-2.

Table 1. UV data on glycosides and aglycone from the rhizomes and roots of *A.calamus*

λ_{max} (nm)	Glycoside	Aglycone
MeOH	265,350	266,320,370
+NaOMe	275,390	278,316,420
+$AlCl_3$	275,306sh,349,401	268,303,350ash,424
+$AlCl_3$ / HCl	270,305sh,351,402	269,302sh,352,420
+NaOAc	273,366	274,386
+NaOAc-H_3BO_3	265,268,351	267,320,371

Table 2. Rf (x100) values of the constituents from the rhizome and roots of *A.calamus* (whatman No.1 ascending 30±2°C)

Compound	Developing solvents*							
	a	b	c	d	e	f	g	h
Flavonol from Et_2O fraction	-	-	6	19	51	93	67	62
Kaempferol (Athentic)	-	-	5	19	50	92	67	63
Flavonol glycoside from EtoAc fraction	40	43	56	73	80	53	65	85
Kaempferol 3-O-rutinoside	40	44	57	72	80	53	66	84

* Solvent key

a-H2O, b-5%aq.HoAc, c-15%aq.HoAc, d-30%aq.HoAc, e-60%aq.HoAc, f-BAW n-BuOH:H_2O=4:1:5, g-water saturated phenol, h-HoAc:Cone HCl:H_2O=30:3:10. The flavonoid spots were localized on Pc by fuming with Mt_3

EtOAc fraction

The residue from EtOAc fraction was taken up in a small quantity of Me_2Co and left in an ice-chest for 3 days. The yellow solid that separated was filtered and recrystallized from aq.MeOH, when yellow needles (m.pt.222-224°C yield-0.1%) were obtained. It develop a red colour with Mg-HCl, yellow with NaOH and deep yellow with NH_3, appeared yellow under UV light with and without NH_3 and responded to Wilson's Boric acid test, molich's test and Gibb's test.

Hydrolysis of the glycoside

Solution of the glycoside (100mg) in hot MeOH (10ml), an equal volume of H_2SO_4 (10%) was added and the mixture was gently refluxed at 100°C for 2 hrs. The excess of alcohol was distilled off in vacuo and the resulting aqueous solution was extracted with Et_2O.

Identification of the aglycone (Kaempferol)

The residue from Et_2O fraction was taken up in Me_2Co and left in ice-chest for a few days when an yellow solid was obtained. It was subjected to colour reactions described under Et_2O fraction.

Test for flavonoids- Shinoda's test

Methanolic extract with few ml of alcohol was heated with magnesium and then concentrated HCl was added under cooling. Appearance of pink colour indicates the presence of flavonoids.

RESULTS AND DISCUSSION

Kaempferol and its 3-O-rutinoside were isolated from root and rhizomes of *A.calamus* (table 1 and 2). The UV spectrum of the flavonolaglycone obtained from the Et_2O fraction showed two major peaks at 266nm and 370nm, which showed a flavonol skeleton. A bathochromic shift of 50nm (Band I) on the addition of NaOMe revealed the presence of 4'-OH group in the B-ring. A shift of +50nm (Band I) on the addition of $AlCl_3$-HCl showed the presence of a free 5-OH in the A ring. The presence of free –OH at C-7 was ascertained by a shift of +8nm (Band II) on the addition of NaOAc. The $AlCl_3$ spectrum was exactly same as that of ($AlCl_3$-HCl) revealing the absence of catechol type of substitution in B ring. The aglycone was identified as kaempferol. This was further confirmed by m.pt, Rf and comparison with an authentic sample (Table 3).

The glycoside from EtOAc fraction has λ_{max}at 350nm (Band I) suggesting a 3-substituted flavonol skeleton. It yielded on hydrolysis (10% H_2SO_4, 100°C, 2hrs) kaempferol. The positive response to Wilson's Boric Acid test indicated the presence of a free-OH at C-5. This was also supported by a bathchromic shift of 40nm (Band I) in its $AlCl_3$ spectrum. A bathochromic shift of 40nm (Band I) in its NaOMe spectrum suggested the presence of free-OH at C-4. A shift of +8nm (Band II) on the addition of NaOAc indicated the presence of a free –OH a free at C-7. The absence of any expected shift in NaOAc-H_3BO_3 spectrum (Band I) when compared with that of NaOAc showed the absence of orthodihydroxy groups in the B ring.

Plant growth promotion by rhizomicroorganisms may be due to the production of growth hormone and vitamin production, nutrient release from soil organic matter or increased uptake and translocation of

Table 3. Qualitative analysis of various solvent extracts of flavonoids of mycorrhizal and non mycorrhizal roots and rhizomes of *A.calamus*

Inoculation treatment	Petroleum ether extract		Benzene extract		Chloroform extract		methanol extract		aqueous extract	
	root	Rhizome	root	rhizome	root	rhizome	root	rhizome	root	rhizome
Mycorrhizal	+	+	+	+	+	+	+	+	+	+
Non mycorrhizal	+	+	+	+	+	+	+	+	+	+

minerals (Chang *et al.,* 1986; Azcon,1989). The residue from Et_2O fraction was taken up in Me_2Co and left in an ice-chest for a week. An yellow solid that separated was filtrated and recrystallized from methanol when pale yellow needles were obtained (m.pt.277-279 ^oC, yield 0.05%). It was readily soluble in alcoholic solvents and sparingly soluble in hot water. It develop a red colour with Mg-HCl and yellow colour with NH_3. It responded to Harhammer-Hansel, Wilson's Boric acid and Gibbs tests (Gerdemann and Nicolson, 1963).

The percent of alkaloids, flavonoids and oil contents obtained in the present study was maximum in the treatment of the dual inoculation. It could be very well noticed that the oil content, flavonoid and an alkaloid content of vasambu plants with dual inoculation proved better than the uninoculated plants. The increase is attributed to the production of hormones (Allen *et al*., 1980; Azcon *et al*., 1989).

Similar results were obtained by Jones Nirmalnath and Sreenivasa (1993) in the sunflower plants inoculated with *G.fasciculatum* and *Pseudomonas striata* and Jayanthi and Bagyaraj (1998) examined the influence of *G.mosseae* and PGPRs *T.harzianum* and B.coagulans on growth and nutrition of microprobagated sugarcane plantlets. Similar results also have been obtained by Krishna Naik *et al*., (1998) using *G.fasciculatum* and *B.coagulans* in the essential oil bearing grass, *Citronella java* and Maheswari *et al.,* (1991) using Azotobacter in the medicinal grass plant, palmarosa. Ratti and Janardhanan (1996) observed the dual inoculation of Palmarosa with *Glomus aggregatum* and *Azospirillum brasilense* increased the growth, percent oil content and flavonoids significantly than compared to uninoculated plants.

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