RESEARCH PAPER

Asian Journal of Bio Science (April, 2010) Vol. 5 Issue 1 : 69-72

Effect of rhizobacterial inoculation on withaferin - A content of ashwagandha (var. Jawahar 20) roots

H. GOPAL AND K. KUMUTHA

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, COIMBATORE (T.N.) INDIA

(Accepted : February, 2010)

Plant growth promoting rhizobacteria *viz.*, *Azospirillum, Azotobacter, Bacillus* and *Pseudomonas* were isolated from rhizosphere soil and roots of ashwagandha plants collected from various locations in Tamil Nadu. The isolated strains were characterized by morphological, physiological and biochemical tests and were examined for nitrogen fixation, phosphate solubilization, phytohormone production, siderophore production and antagonistic activity. A pot culture experiment was conducted at the dept of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The results revealed that combined inoculation of *A. lipoferum* - AAs-11, *Azotobacter* - AAz-3, *Bacillus* - APb-1 and *Pseudomonas fluorescens* - APs-1 enhanced the biochemical constituents of ashwagandha such as chlorophyll, protein and total alkaloid contents, especially Withaferin-A.

Key words : Rhizobacteria, Aswagandha, Withaferin-A

INTRODUCTION

The indigenous systems of medicine namely Siddha, Ayurveda and Unani have been in existence for several centuries. The WHO has estimated that over 80 per cent of the world population meets their primary health care needs through traditional medicine (Lambert, 1997). Ashwagandha is used as a tonic in geriatrics, being efficacious in relieving hand and limb tremors of people at old age (Atal *et al.*, 1975). It has been equated to ginseng (Panax ginseng) of China and is popularly known as the "Indian Ginseng". The most important pharmacological use of ashwagandha is as adaptogen with antistress antioxidant, antitumor, anti-inflammatory, mind boosting and has rejuvenating properties (Singh *et al.*, 1990). The biofertilizers are ecofriendly and low cost technology and their application may play a major role in soil fertility, nutrient transformation, crop sanitation and sustainability. The rhizobiocoenosis is an important biological process that plays a major role in satisfying the nutritional requirement of these crops. Studies on the rhizobacterial population in the rhizosphere region and testing the suitability of the isolated rhizobacteria as seed and soil inoculant will be highly useful in improving the productivity and quality of this commercially important medicinal plant.

MATERIALS AND METHODS

A pot culture experiment was conducted during the year 2004 at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore (T.N.) to study the effect of combined inoculation of rhizobacteria on growth, yield and quality of ashwagandha (var. Jawahar 20). The rhizobacterial isolates *viz.*, *Azospirillum lipoferum*-AAs-11, *Azotobacter*-AAz-3, *Bacillus*-APb-1 and *Pseudomonas fluorescens*-APs-1 were prepared as carrier based inoculants used for this study. The pots were filled with potting mixture (soil + sand + FYM) and the rhizobacteria treated seeds were sown at 25 seeds per pot and finally 5 seedlings were maintained. The experiment was conducted in completely randomized block design with three replications. The analysis of the alcoholic extracts of the root samples were carried out by the method suggested by Velde *et al*. (1983) by using the high performance thin layer chromatography (HPTLC) available at J.S.S. College of Pharmacy, Ooty.

Sample preparation :

Ten gram of root samples were dissolved in 100 ml of methanol and filtered through Whatman No. 42 filter paper. The methanol extracts of root samples were used for direct application on the HPTLC plate.

Preparation of the standard solution :

Withaferin-A at the rate of 5.4 mg was dissolved in 5 ml of methanol so as to get a concentration of 1.08 mg ml^{-1}.

Selection of HPTLC plates :

Pre-coated silica gel GF_{254} plates with alumina support in size of 20 x 20 cm were used for the present study.

Application of sample :

The Linomat allows sample application in narrow bands by a spray on technique under an inert gas blanket. All the sample solutions were applied as a thin band of 6mm width containing a sample volume of 5m1 for the standard marker compounds and 10 ml for the sample solutions into a suitable track on the plate by using Camag Linomat IV, which is a microprocessor controlled and programmable applicator.

Mobile phase :

Toluene: ethyl acetate: formic acid (50:15:5)

Chromatographic development :

All the plates after drying were developed in previously saturated Camag Twin trough chamber using the above mentioned mobile phase. After proper development, the plates were removed from the chamber and air dried with the help of hand drier to effect faster removal of the mobile phase.

Densitometric chromatogram development :

For densitometric measurements of a thin layer chromatogram, it's separation tracks were scanned with a light beam in the form of a slit selectable in length and width. Diffusely reflected light is measured by photosensor. Densitometric measurements can be made by absorbance or by fluorescence. For scanning by fluorescence, the substances are excited by UV light, most often at 366nm. In order to avoid systematic errors, scanning should always be done in or against the direction of chromatography. The decrease in the light reflectance due to adsorbed compounds gives rise to a signal in the detectors. The signal thus generated is amplified and transmitted to a recorder where the spots, by absorbing some of the light, cause a signal to lessen and a resultant peak or dip is printed by the detector. The areas included in those peaks measured and relate to amount of material in the spot.

The scanner is linked to the personal computer, from which all commands are passed to the scanner. The scanner transmits all measurement data in digital form to the computer for further processing. For the present study, the developed chromatograms were evaluated by using Camag TLC scanner 3 at a wave length of 250nm. The sequence in the quantitative evaluation of a chromatogram is: raw data acquisition - integration - calibration and calculation result generating the analysis report. For all the samples, integration calibration spectra were recorded and the total area included in the peak was observed. The quantity of Withaferin-A in the sample was calculated by comparing with the area given by the standard and expressed as mg 100 g^{-1} of root.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below :

Alkaloid content :

The results on the alkaloid content of ashwagandha root are presented in Table 1. Inoculation with rhizobacteria significantly influenced alkaloid content of ashwagandha. The alkaloid content of roots varied from 44.00 to 87.00 mg $plant^{-1}$ in various treatments. Among the treatments, the combined application of *Azospirillum lipoferum*-AAs-11, *Azotobacter*-AAz-3, *Bacillus*-APb-1 and *Pseudomonas fluorescens*-APs-1 recorded the maximum total alkaloid yield of 87.00 mg $plant^{-1}$ followed by triple inoculation of *Azospirillum lipoferum*-AAs-11, *Bacillus*-APb-1 and *Pseudomonas fluorescens*-APs-1 (71.00 mg $plant^{-1}$). Among the individual inoculants, *Azospirillum lipoferum*-AAs-11 inoculated roots recorded maximum alkaloid (56 mg $plant^{-1}$) than *Azotobacter*-AAz-3, *Bacillus*-APb-1 and *Pseudomonas fluorescens*-APs-1. The roots of uninoculated plants recorded the least alkaloid content (44 mg $plant^{-1}$).

The Withaferin–A content was estimated in few selected treatments. The Withaferin-A content of ashwagandha roots ranged between 40.40 and 110.00 mg 100 g^{-1} of roots in various treatments as measured by high performance thin layer chromatography (HPTLC).

Inoculation of rhizobacteria either alone or in various combinations increased Withaferin –A content in

Table 1 : Effect of rhizobacterial inoculation on total alkaloid content of ashwagandha (var. Jawahar 20) roots

Treatments	Total alkaloid (%)	Total alkaloid yield (mg plant-1)
T_1 – *Azospirillum* (AAs-11)	1.18	56
T_2 – *Azotobacter* (AAz-3)	1.12	48
T_3 – *Bacillus* (APb-1)	1.13	49
T_4 – *Pseudomonas* (APs-1)	1.15	51
T_5 – T_1 + T_2	1.20	59
T_6 – T_1 +T_3+T_4	1.29	71
T_7 – T_2 +T_3+T_4	1.26	67
T_8 – T_1+T_2+T_3	1.23	62
T_9 – T_1+T_2+T_3+T_4	1.42	87
T_{10} – Uninoculated control	1.10	44
S.E. ±	0.10	5.33
C.D. (P=0.05)	0.22	11.13

Table 2 : Effect of rhizobacterial inoculation on Withaferin – A content of ashwagandha (var. Jawahar 20) roots by HPTLC

Treatments			Withaferin – A content (mg 100 g^{-1} of roots)
T_1	-	*Azospirillum* (AAs-11)	44.80
T_2	-	*Azospirillum* (AAs-11) + *Azotobacter* (AAz-3)	57.80
T_6	-	*Azospirillum* (AAs-11) + *Bacilluss* (APb-1) + *Pseudomonas* (APs-1)	66.42
T_9	-	*Azospirillum* (AAs-11) + *Azotobacter* (AAz-3) + *Bacillus* (APb-1) + *Pseudomonas* (APs-1)	110.00
T_{10}	-	Uninoculated control	40.40

ashwagandha (Table 2). Maximum Withaferin-A content was recorded in the treatment receiving all the four rhizobacterial inoculants together (110.00 mg 100 g^{-1}) followed by triple inoculation of *Azospirillum lipoferum*-AAs-11, *Bacillus*-APb-1 and *Pseudomonas fluorescens*-APs-1 (66.42 mg 100 g^{-1} root) or dual inoculation of *Azospirillum lipoferum*-AAs-11 and *Azotobacter*-AAz-3 (57.80 mg $plant^{-1}$). The individual *Azospirillum* inoculation also recorded higher Withaferin-A content (44.80 mg 100 g^{-1} root) than the uninoculated control (40.40 mg 100 g^{-1}root).

The inoculation of the mixed inoculant stimulated the population of *Azospirillum, Azotobacter, Bacillus* and *Pseudomonas* in the rhizosphere. The increased activity of the microbial population in the rhizosphere might have influenced the nutrient uptake, biomass and grain yield than single inoculation. Similar results have also been reported in pearl millet (Ramamourty, 1982); rice (Lakshmipriya, 1997) and cotton (Sugunarani, 2000). Alagawadi and Gaur (1988) noted that dual inoculation of PSB with *A.brasilense* increased the root associated acetylene reduction activity and crop yield in sorghum plants over single inoculation of *Azospirillum*. Positive interaction of *Azospirillum* with phosphobacteria has been reported in many cereals and vegetable crops. Inoculation of *A. lipoferum* along with PSB *Bacillus* (SB1) recorded 17.5% yield increase in rice over uninoculated control (Lakshmipriya, 1997).

Combined inoculation of all the four rhizobacteria recorded maximum nitrogen and phosphorus content in plants and soil, indicating the positive effect of combined inoculation in augmenting the availability of nitrogen and phosphorus to plants. The result is in agreement with the findings of Alagawadi and Gaur (1992). Plants receiving dual inoculation of *A. lipoferum* and phosphobacteria recorded maximum N and P content than the individual inoculation (Lakshmipriya, 1997). The increased activity of rhizobacteria in the rhizosphere might have influenced the nitrogen fixation, phosphorus solubilization, nutrient uptake, biomass and grain yield than the single inoculation. Belimov *et al*. (1995) found through isotope studies that combined inoculation of nitrogen fixing and phosphate solubilizing organisms enhanced the absorption of N and P in barley plants.

Increase in plant nitrogen content due to *Azospirillum* inoculation has been observed by several workers in many crops (Raverkar and Konde, 1988; Aswah and Shahby, 1993 and Dobbelaere *et al.*, 2001). Since plants inoculated with nitrogen fixers had maximum N content, it is reasonable to think that the inoculation might have enhanced N uptake by the plants due to increased availability in the rhizosphere by the activity of the inoculated bacteria. Increased total N content due to the *Azospirillum* inoculation was also reported by Rao *et al*. (1983) in paddy.

In general, production of growth promoting substances like gibberellins and auxins by the rhizobacteria enhanced proliferation of root system which in turn enhanced mineral uptake (N, P and K) and consequently increased dry matter accumulation (Okon, 1985 and Lakshmipriya, 1997). The positive effects observed in the present study may be attributed to considerable quantity of indole acetic acid (91.2, 72.6 and 34.8 µg 25 ml^{-1} by AAs-11, APs-1 and APb-1, respectively) and gibberellic acid (5.71, 5.61 and 4.12µg 25 ml^{-1} by AAs-11, APs-1 and APb-1, respectively) production besides nitrogen fixation by the rhizobacteria. The rhizobacterial inoculation besides increasing yield also enhanced the alkaloid content of roots, especially Withaferin-A. This alkaloid is mainly responsible for the various pharmaceutical properties of ashwagandha. The known and unknown activities of the rhizobacteria may be attributed to the increased alkaloid content of ashwagandha.

REFERENCES

Alagawadi, A.R. and Gaur, A.C. (1988). Interaction between *Azospirillum brasilense* and phosphate solubilizing bacteria and their influence on yield and nutrient uptake of sorghum (*Sorghum bicolor* L.). *Zentralbl. Mikrobiol.*, **143**: 637-643.

Alagawadi, A.R. and Gaur, A.C. (1992). Inoculation of *Azospirillum brasilense* and PSB on yield of sorghum in dryland. *Tropical Agric.*, **69**: 347-350.

Aswah, E.M.R and Shahby, A.F. (1993). Associative effect of *Azospirillum* on vitamin C, chlorophyll content and growth of lettuce under field conditions. *Ann. Agric. Sci.*, **38**: 423-434.

Belimov, A.A., Kojemiakor, A.P. and Chuvarliyeva, C.V. (1995). Interaction between barley and mixed cultures of nitrogen fixing and phosphate solubilizing bacteria. *Plant Soil*, **173** : 29-37.

Dobbelaere, S., Croonenborghs, A., Thys, A., Ptacek, D., Vanderleyden, J., Dutto, P., Labandera-Gonzalez, C., Caballero-Mellado, J., Aguire, J.F., Kapulnik, Y., Brener, S., Burdman, S., Kadouri, D., Sarig, S. and Okon, Y. (2001). Response of agronomically important crops to inoculation with *Azospirillum*. *Australian J. Plant Physiol*., **28** : 871-879.

Lakshmipriya, D. (1997). Studies on phosphate solubilizing bacteria. M.Sc. (Agri.) Thesis, Tamil Nadu Agricultural University, Coimbatore-3. pp. 153.

Okon, Y. (1985). *Azospirillum* as a potential inoculant for agriculture. *TIBTECH*, **3**: 223-228.

Ramamourty, A. (1982). Studies on the interaction between phosphobacterium and nitrogen fixing microorganisms in relation to productivity of pearlmillet and cowpea. M.Sc. (Ag.) Thesis, Tamil Nadu Agricultural University, Coimbatore. pp. 157.

Rao, V.R., Nayak, D.N., Charyulu, P.B.B.N. and Adhya, T.K. (1983). Yield response of rice root inoculation with *Azospirillum*. *Agric. Sci. Camb*., **9**: 118-122.

Raverkar, K.P. and Konde, B.K. (1988). Effect of *Rhizobium* and *Azospirillum lipoferum* inoculation on the nodulation, yield and nitrogen uptake of peanut cultivars. *Plant Soil*, **106**: 249-252.

Sugunarani, K. (2000). Influence of plant growth promoting rhizobacteria on the population dynamics of the rhizosphere microflora, growth and development in cotton (*Gossypium hirsutum*). Ph.D. Thesis,Tamil Nadu Agricultural University, Coimbatore. pp. 183.