Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 4, 2013

Research Article

EFFECT OF *Pseudomonas fluorescens* ISOLATED FROM VARIOUS AGRO CLIMATIC ZONES OF KARNATAKA ON *Rauwolfia serpentina*

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Received: 01 Aug 2013, Revised and Accepted: 28 Sep 2013

ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize in plant roots and increase plant growth and yield. The aim of this project was to study the physiological and molecular characterization of *Pseudomonas fluorescens* isolated from different agro climatic zones of Karnataka and its effects on the medicinal plant Sarpagandha *(Rauwolfia serpentina)*. *P. fluorescens* strains from 10 different agro climate zones were isolated, identified and confirmed using standard synaptic keys. Molecular diversity of these isolates was characterized by RAPD marker analysis. The effect of the isolated organisms on the physical and biochemical parameters of Sarpagandha (*Rauwolfia serpentina*) was studied. The parameters such as height, number of leaves, fresh and dry weight of the roots and shoots, were measured along with the control (uninoculated plants). Biochemical parameters like chlorophyll content, total sugar and protein content in root and shoot were estimated too. The results showed that *P. fluorescens* has profound effect on the growth as well as the nutrient content of the plants. The genetic relatedness of the 10 isolates was studied and these formed 2 groups based on the phylogenetic tree obtained. The genetic variations however had no relationship with the effect on plant growth and nutrition. From the study, it could be concluded that the *Pseudomonas fluorescens* isolates of zone-3 and zone-6 showed maximum effect on growth and nutrient content of Sarpagandha compared to uninoculated control.

Keywords: Chlorophyll, *Pseudomonas fluorescens, PGPR, Rauwolfia serpentina*

INTRODUCTION

Bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). Their effects can occur via local antagonism to soil-borne pathogens or by induction of systemic resistance against pathogens throughout the entire plant [1]. Plant growth promoting rhizobacterial (PGPR) strains can be used as inoculants for crops grown in stressed ecosystems [2]. The ability of rhizobacteria to produce siderophores and metabolites contributing to antibiosis has been the focus of many studies dedicated to investigating PGPR [3]. The PGPR is known to facilitate the plant growth through nitrogen fixation; solubilization of insoluble phosphorus; production of compounds like siderophores, phytohormones, antibiotic and antifungal metabolites; and induced systemic resistance [4]. During the past couple of decades, the use of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported [5].

Among PGPR, PSB (Phosphate Solubilizing bacteria) supply P to plants [6]. Bacteria of the groups *Bacillus* and *Pseudomonas* have proven to be the most powerful phosphate solubilizing bacteria. Fluorescent *pseudomonads* are non- pathogenic rhizobacteria and several isolates *of Pseudomonas fluorescens, P. putida, P. aeruginosa and P. aureofaciens* suppressed the soil borne pathogens through different proposed mechanisms including rhizosphere colonization, antibiosis and iron chelation by siderophore production [7].

Sarpagandha (*Rauwolfia serpentina*) is a medicinal plant par excellence, producing useful alkaloids like reserpine. Reserpine is an indole alkaloid and is important constituent of *Rauwolfia* which is reported to posses anti hypertensive and tranquilizing activity [8]. It has been reported to contain 50 indole alkaloids that are mainly localized in the root bark. Among these alkaloids, reserpine, yohimbine, serpentina, deserpidine, ajmalicine, ajmaline, recinnamine, ajmalidine, sarpagine, raucaffricine, etc. are the rich source found in root of *R. serpentina* [9]. Various parts of this plant are used to treat human ailments, in alternative systems of medicine. Sarpagandha is a threatened species found in the subtropical regions. Seed propagation is considered to be the best method for raising commercial crop, though seed production is highly variable and low [10].

MATERIALS AND METHODS

The present investigation was carried out to isolate and identify *Pseudomonas fluorescens* from soil of different agro climatic zones of Karnataka and to characterize those using RAPD markers.

Sample collection and Isolation of *P. fluorescens*

The soil samples were collected from the different agro climatic zones of Karnataka. Rhizosphere soil was collected in polythene covers and stored in cool place. *P. fluorescens* were isolated by following the enrichment culture technique on King's 'B' agar medium [11]. The isolated colonies were subjected for morphological characterization and biochemical tests. Gelatin liquefaction test, Levan formation test [12], Indole production test [13], catalase and motility test were carried out.

DNA isolation and amplification

DNA was isolated from bacterial isolates grown in Kings 'B' broth by phenol, chloroform and isoamyl alcohol mixture. The isolated DNA was amplified by using four random primers (Table 1).

Table 1: Random Primers used for RAPD

Primer No.	Sequence (5'-3')
Random primer 1	GAG AGC CAA C
Random primer 2	GTT TCG CTC C
Random primer 3	GTA GAC CCG A
Random primer 4	AAG AGC CCG T

1.5% Agarose gel was used to resolve the amplification product and the gel was visualized under UV light and documented using Hero Lab Gel Documentation unit.

Analysis of RAPD data

The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data (Presence or absence) was subjected to cluster analysis-using STATISTICA. The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm. The dissimilarity matrix was developed using Squared Euclidean Distance (SED), which estimated all the pair wise differences in the amplification product [14]. Only clear and unambiguous bands were taken in to account and the bands were not scored if they were faint or diffused, as such fragments posses poor reproducibility. The band

sizes were determined by comparing with the 500 bp DNA ladder, which was run along with the amplified products. The Genetic distance was computed as:

$$\Sigma^{n}=1\ dj^{2} \text{ where } dj = (Xik - X_{jk})$$

Where X_{ik} refers to binary code of i^{th} tree for allele "k" and X_{jk} refers to the binary code of the j^{th} tree for allele "k". Dendrogram was computed based on Ward's method of clustering, using minimum variance algorithm [15].

Inoculum preparation

Pseudomonas fluorescens isolates were grown separately in a 250ml flask containing 100ml King's B broth for 24 hours. The grown cultures were homogenized and 3ml of each of the solution was given to each plant sapling.

Physical and Biochemical Parameters

Plant saplings were obtained from division of horticulture, University of Agricultural sciences, GKVK, Bangalore. Plant saplings of the plant were planted on pots containing sterile sand: soil mixture (1:1). The plants were allowed to grow for 60 days by watering as per the requirement. The physical parameters namely plant height, number of branches, root fresh and dry weight and shoot fresh and dry weight were determined on 15^{th}, 30^{th} and 45^{th} days. Root and shoot dry weights were calculated by drying the harvested roots or shoots in an oven at 60^{o}C for 4 days to attain constant weight and then the root dry weight were recorded and expressed as grams/plant and expressed as grams/plant. The biochemical parameters namely chlorophyll content [16], total soluble proteins [17] and total soluble sugar [18] were estimated on 60^{th} day.

Chlorophyll content (mg / g fresh weight) were calculated by the following formulae [19]

$$\text{Chlorophyll 'a'} = \frac{12.7\ (OD\ at\ 663) - 2.69\ (OD\ at\ 645) \times Volume}{1000g \times Weight\ of\ leaves\ (g)}$$

$$\text{Chlorophyll 'b'} = \frac{22.9(OD\ at\ 645) - 4.68\ (OD\ at\ 663) \times Volume}{1000g \times Weight\ of\ leaves\ (g)}$$

$$\text{Total chlorophyll} = \frac{20.2\ (OD\ at\ 645) + 8.02\ (OD\ at\ 663) \times Volume}{1000g \times Weight\ of\ leaves\ (g)}$$

Statistical analysis

The data obtained in the pot experiments were subjected to one way analysis of variance using MSTAT-C software.

RESULTS AND DISCUSSION

Isolation and identification of the bacteria

Fluorescent bacteria were isolated on King's 'B' agar medium from soils of different agroclimatic zones of Karnataka (Table 2) and characterized on King's 'B' agar medium which is selective medium for isolation of *Pseudomonas fluorescens* [20]. There were 10 isolates obtained from 10 zones by isolating on King's B medium. All isolates were found to be Gram-negative, and rod shaped [21]. They formed bluish green smooth colonies which fluoresced under UV light and produced water soluble, fluorescent bluish green pigmentation in King's 'B' broth. All isolates were found to exhibit positive result to gelatin liquefaction as it was indicated by the production of yellowish or bluish green fluid on the surface of gelatin agar medium, produced slimy colonies due to Levan formation [22], were found indole negative as they did not form a red layer at the top of tryptophan broth medium and positive to catalase as indicated by formation of air bubbles on addition of hydrogen peroxide [20]. Isolates of all 10 zones were also found positive to motility as indicated by diffused growth spread throughout the medium (Table 3). The 10 isolates obtained from samples of different area have been mentioned as either Zone -1 to Zone – 10 or Z1 to Z10 in results.

Table 2: List of isolates and their samples

Isolate	Zone of Collection	Name
Isolate 1	North Eastern Transition Zone	NETZ or zone 1
Isolate 2	North Eastern Dry Zone	NEDZ or zone 2
Isolate 3	Northern Dry Zone	NDZ or zone 3
Isolate 4	Central Dry Zone	CDZ or zone 4
Isolate 5	Eastern Dry Zone	EDZ or zone 5
Isolate 6	Southern Dry Zone	SDZ or zone 6
Isolate 7	Southern Transition Zone	STZ or zone 7
Isolate 8	Northern Transition Zone	NTZ or zone 8
Isolate 9	Hill Zone	HZ or zone 9
Isolate 10	Coastal Zone	CZ or zone 10

Table 3: Morphological characters of *Pseudomonas fluorescens*

Sl. No	Isolates	Colony Morphology			Cell Shape	Gram Reaction	Epifluorescence
		Colour	Shape	Nature			
1	North Eastern Transition Zone	Green	Irregular	S	Rod	Negative	+++
2	North Eastern Dry Zone	L. Green	Irregular	N.S.	Rod	Negative	++
3	Northern Dry Zone	L. Green	Irregular	N.S.	Rod	Negative	++
4	Central Dry Zone	Green	Round	N.S.	Rod	Negative	++
5	Eastern Dry Zone	L. Green	Round	N.S.	Rod	Negative	++
6	Southern Dry Zone	L. Green	Round	N.S.	Rod	Negative	+++
7	Southern Transition Zone	L. Green	Round	N.S.	Rod	Negative	++
8	Northern Transition Zone	Green	Round	N.S.	Rod	Negative	+
9	Hill Zone	L. Green	Round	N.S.	Rod	Negative	++
10	Coastal Zone	L. Green	Round	N.S.	Rod	Negative	+++

(NS-Non spreading; S- Spreading; L.green = Light green; + + + =High fluorescence; + + = Medium fluorescence; + =Weak fluorescence)

In the present study, *Pseudomonas* was isolated from different agroclimatic zones of Karnataka. This showed that the microorganism is a common inhabitant of rhizosphere of many plant species in different areas. Previous studies have shown that the *Pseudomonas* are very common in many parts of the world including tropical, temperate or subtropical region and in association with different plant viz. cereals, vegetables, etc [20, 22].

After the preliminary screening 10 *Pseudomonas fluorescens* isolates from each Zones viz., NETZ, NEDZ, NDZ, CDZ, EDZ, SDZ, STZ, NTZ, HZ and CZ and they were examined for their performance under Green house condition.

Morphological and biochemical parameters

The plant height was significantly increased in the inoculated plants at 15, 45, and 60 days after planting, compared to control. Maximum height of 91.3 cm was observed in the plants inoculated with zone-6 (SCZ) isolate (Table 4). This indicated that the Zone-6 isolate is more efficient in enhancing the plant height compared to others. Similar results were observed by Akhtar et al [23] and Yadav et al. [24]. Umashankari and Sekar [25] used various bioformulations of *Pseudomonas fluorescens (PF-3)* and *Paenibacillus polymyxa (B-19)* on *Oryza sativa* and found that there was increase in plant height in all formulations compared to the control.

In all the inoculated treatments number of leaves per plant increased significantly compared to the uninoculated plants at all the growth intervals. The Zone-6 isolate of *Pseudomonas fluorescens* inoculated plants produced significantly highest number of leaves of 118 among all the isolates (Table 5). This indicated the superiority of Zone-6 isolates as compared to others. Similar results were observed by Hammad et al [26]. Yolcu et al [27] studied the effects of certain strains of PGPR and found that the number of leaves increased significantly at %1 level.

Plant Biomass

The total fresh weight (shoot and root) of Sarpagandha was significantly higher in the plants inoculated with *P.fluorescens* isolates compared to uninoculated control. Maximum total fresh weight of 30.86g for shoot and 25.99g for root was recorded in the zone-6 isolate (Figure1 and 2). This suggested that the zone-6 isolate is most efficient in enhancing the biomass compared to others. The total dry weight (shoot and root) of Sarpagandha was significantly higher in the plants inoculated with *P.fluorescens* isolates compared to uninoculated control. Maximum total dry weight of 17.01g for shoot and 16.41g for root was recorded in the zone-6 isolate (Figure1 and 2). This suggested that the zone-6 isolate is most efficient in enhancing the biomass compared to others. Similar result was also reported by. Similar results were observed by Akhtar et al [23]. Adjanohoun et al [28] studied the effects of 15 maize fields isolated PGPR on nondegraded reddish ferrous field grown maize in southern Benin and each of these showed an increase in fresh weights of roots and shoots compared to the control. Umashankari and Sekar [25] used various bioformulations of *Pseudomonas fluorescens (PF-3)* and *Paenibacillus polymyxa (B-19)* on *Oryza sativa* and found that there was increase in shoot and root dry weight in all formulations compared to the control.

Chlorophyll estimation

There was significant increase in the chlorophyll content in *P.fluorescens* treated plants as compared to control. Zone-6 treated plants recorded highest chlorophyll content of 1.27mg per plant (Figure 3). Also a SEM of 0.015 for chlorophyll A and 0.004 for Chlorophyll B and CD of 0.046 for chlorophyll A and 0.013 for Chlorophyll B were obtained. Similar results were observed by Akhtar et al [23].

Total sugar content

There was significant increase in the total sugar content in *P.fluorescens* treated plants as compared to control. Zone-6 treated plants recorded highest sugar content of 3.306 mg per plant (Figure 4). Also a SEM of 0.056 for shoot and 0.006 for root and CD of 0.165 for shoot and 0.020 for root were obtained. Similarly Prathibha and Siddalingeshwara [29] noticed increase in total carbohydrate as influenced by *Bacillus subtilis* and *Pseudomonas fluorescencs* on seed of sorghum as compared to control.

Total protein content

There was significant increase in the total soluble protein content in *P .fluorescens* treated plants as compared to control.Zone-6 treated plants recorded highest protein content of 20.31 mg per plant (Figure 5). Also a SEM of 0.547 for shoot and 0.065 for root and CD of 1.606 for shoot and 0.191 for root were obtained. Similarly Prathibha and Siddalingeshwara [29] noticed increased total soluble protein content as influenced by *Bacillus subtilis* and *Pseudomonas fluorescens* on seed of sorghum as compared to control.

Table 4: Effect of *P.fluorescens* on the height of *R. serpentina*

Inoculum	Height of plants (cm)			
	15th Day	30th Day	45th Day	60th Day
Control	11.7	13.7	17.6	9.6
Zone 1	14.9	23.1	31.1	39.6
Zone 2	17.4	24.2	29.6	35.3
Zone 3	18.9	34.2	42.6	82
Zone 4	12.8	22.0	30.4	66.3
Zone 5	15.7	24.7	30.4	43.3
Zone 6	**20.5**	**28.2**	**39.5**	**91.3**
Zone 7	16.9	22.0	27.1	41.3
Zone 8	14.7	26.5	41.4	60
Zone 9	17.2	28.9	41.8	48.3
Zone 10	15.9	23.9	32.3	36
SEM	0.2954	0.70563	0.4814	1.1221
CD	0.8664	2.06954	1.4119	3.2912

Table 5: Effect of *P.fluorescens* on number of leaves of *R. serpentina*

Inoculum	Number of Leaves			
	15th Day	30th Day	45th Day	60th Day
Control	6	7	10	11
Zone 1	19	29	40	48
Zone 2	17	24	35	37
Zone 3	21	49	82	100
Zone 4	19	44	66	76
Zone 5	12	27	43	54
Zone 6	**23**	**78**	**91**	**118**
Zone 7	15	29	41	53
Zone 8	17	43	60	84
Zone 9	18	37	48	71
Zone 10	14	28	36	40
SEM	0.4568	0.7224	1.1221	1.3657
CD	1.3400	2.1188	3.2912	4.0056

(SEM - Standard error mean: CD - Critical difference)

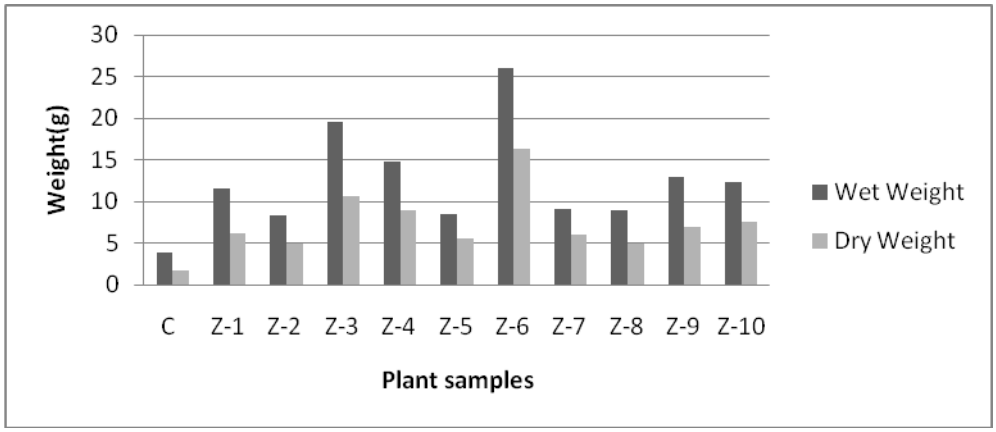

Fig. 1: Effect of *P.fluorescens* on root biomass of *R. serpentina*

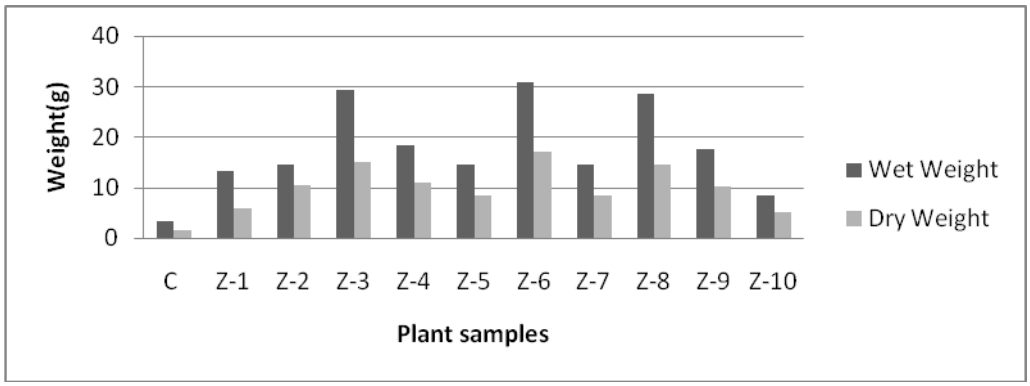

Fig. 2: Effect of *P.fluorescens* on shoot biomass of *R. serpentina*

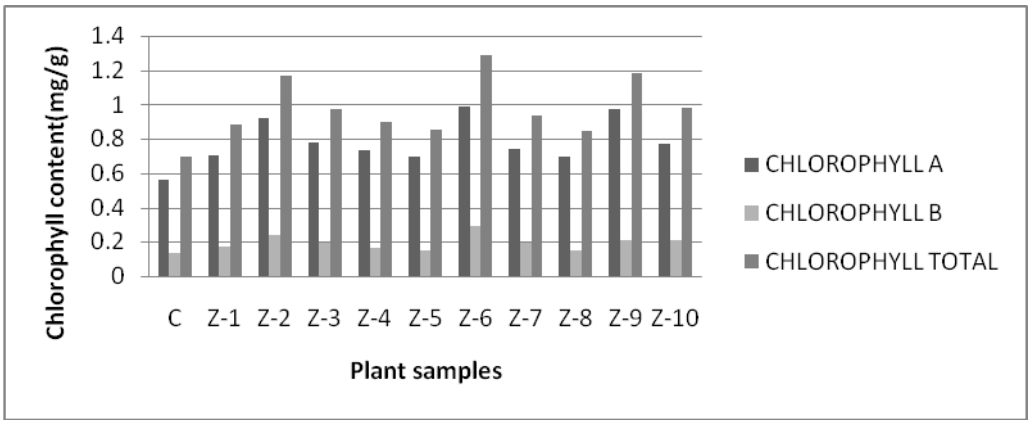

Fig. 3: Effect of *P.fluorescens* on chlorophyll content of *R. serpentina*

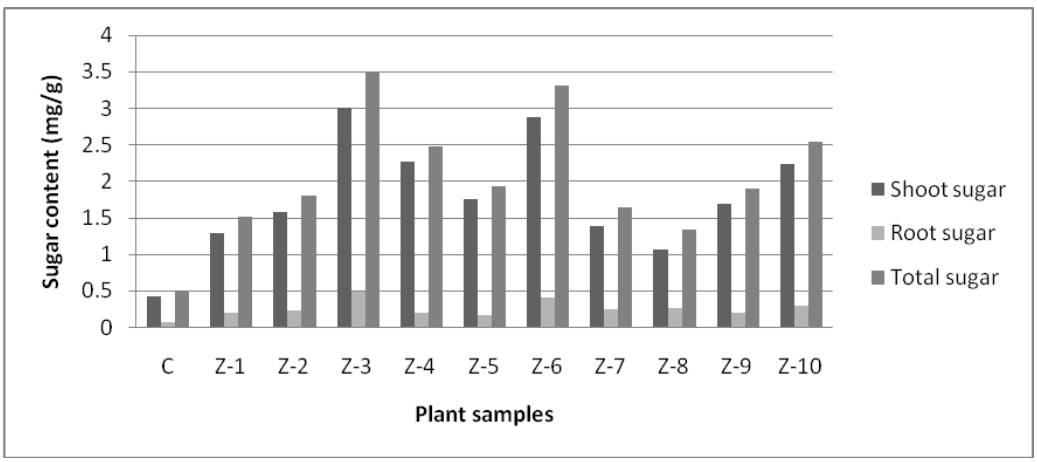

Fig. 4: Effect of *P.fluorescens* on sugar content of *R. serpentina*

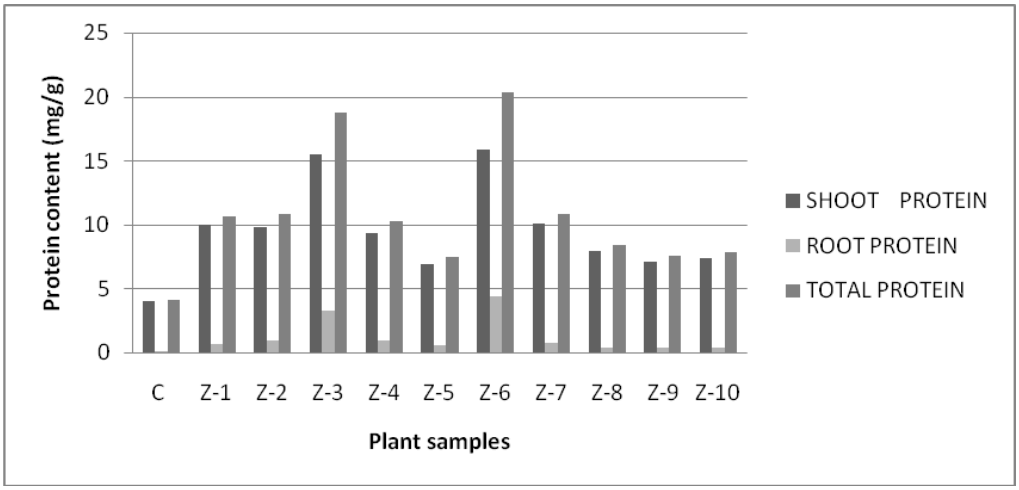

Fig. 5: Effect of *P. fluorescens* on protein content of *R. serpentina*

Molecular identification of *P. fluorescens*

Four random primers were selected for fingerprinting and diversity analysis of *P. fluorescens* isolates. A total of 40 bands (details not shown) were scored, out of which 37 bands were found to be polymorphic (92.5%) and they seemed to be quite high within species. The products of RAPD when subjected to Gel electrophoresis and 40 bands were obtained (Table 6). The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis (Table 6). The scored band data (Presence or absence) was subjected to cluster analysis-using STATISTICA.

The dendrogram (Figure 6) has clearly depicted that all the 10 *P. fluorescens* isolates formed two major clusters. Among the two major groups, isolates from zone 4 (CDZ), zone 5 (EDZ), and zone 6 (SDZ) formed the first group and the isolate from zone 1 (NETZ), zone 2 (NEDZ), zone 3 (NDZ), zone 7 (STZ), zone 8 (NTZ), zone 9 (HZ) and zone 10 (CZ) formed the second group. The dissimilarity matrix for *P. fluorescens* isolates (Table 5) revealed that within the *P. fluorescens* isolates used in the present investigation, the highest dissimilarity was observed between isolate of zone 1 (NETZ) and isolate of zone 4(CDZ) followed by zone 4(CDZ) and zone 9 (HZ) isolates. Least dissimilarity was observed between zone 2 (NEDZ) and zone 3 (NDZ) isolates, followed by zone 3 (NDZ) and zone 10 (CZ) isolates.

Postic et al [30] used 4 primers to study the similarity of selected Pseudomonas spp. isolates on the basis of RAPD analysis and cluster analysis revealed 4 of the 5 strains forming one cluster. Similarly Marinkovic et al [31] obtained dendrogram of *Bradyrhizobium japonicum* strains obtained by RAPD method using four primers and it showed 4 of the 5 strains forming one cluster. Amplification with RAPD primers showed the greatest genetic diversity between strains D216 and 511.

Table 6: List of primers used for genetic variation among the *P. fluorescens* isolates

Primers	No. of amplified fragments	No. of polymorphic bands		No. of Monomorphic bands
		shared	Unique	
Random primer 1	12	07	04	01
Random primer 2	08	04	02	02
Random primer 3	10	06	04	00
Random primer 4	10	10	00	00
Total	40	27	10	03
Percentage		67.50%	25%	7.50%

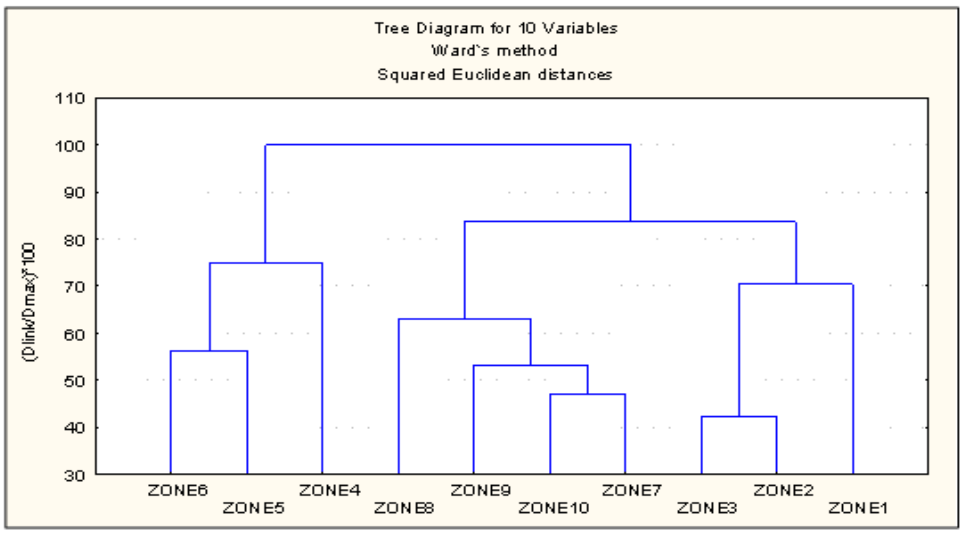

Fig. 6: Dendrogram based on RAPD profile of 10 isolated *P. fluorescens*

CONCLUSION

Pseudomonas fluorescens strains from different agro climatic zones were isolated, identified and confirmed using standard synaptic keys. Molecular diversity of these isolates was characterized by and the RAPD banding pattern of these isolates could easily distinguish the isolates of different zones. Simultaneously the growth response studies using Sarpagandha as host was conducted. From the study, it can be concluded that the *P.fluorescens* isolates stimulated the growth responses of sarpagandha.

ACKNOWLEDGEMENT

Heartfelt thanks to Department of Plant Biotechnology, University of Agricultural Sciences, G.K.V.K Campus, Bangalore, for having given permission to carry out the work by providing all the infrastructure and materials required for the study.

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