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COMPARATIVE STUDY OF PIRIFORMOSPORA INDICA AND SALICYLIC ACID ON THE GROWTH PROMOTIONAL ACTIVITY OF ARTEMISIA ANNUA L

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ABSTRACT

Salicylic acid and *Piriformospora indica* colonized plants were reported as potent enhancers of growth and photosynthetic activity. The present study was conducted to study the comparative alteration of plant growth and photosynthetic activity of the medicinal plant *Artemisia annua* treated with *P. indica* and salicylic acid under *in vitro* condition. The *in vitro* grown plantlets treated with *P. indica* showed a higher value in all the growth parameter and photosynthetic pigments content followed by salicylic acid. The callus showed highest values of fresh weight on salicylic acid treatment.

KEYWORDS: ***Artemisia annua*** **,** ***in vitro*****, callus and plant growth**

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INTRODUCTON

Secondary metabolite, also known as phytochemicls, which are unique sources for food additives, flavours, pharmaceuticals, and industrially important pharmaceuticals[1] are synthesised by plants in particular needs, act in defence purposes to protect the plant from any possible harm in the environment[2]. Secondary metabolites accumulation often occurs in plants subjected to stresses including various elicitors or signal molecules such as calcium, abscisic acid (ABA), salicylic acid (SA), polyamines and Jasmonates (JA), nitric oxide[3]. Production of these compounds are often low (less than 1% dry weight) and depends greatly on the physiological and developmental stage of the plant [4]. Artemisinin, an endoperoxide containing sesquiterpene compound, found in the trichomes of *Artemisia annua* [5], is an anti-malarial drug of immense therapeutic importance. Its yield remains low in natural condition which is around 0.001-1% [6] dry weight and its production is subjected to seasonal and commercial limitations. There is an urgent need to develop high artemisinin content varieties that will produce acceptable yields under favourable and unfavourable environments[7]. Though, diverse strategies are being adopted to enhance artemisinin concentration in *A. annua* by selecting high yielding cultivars or by creating transgenic plants[8] still there is a major challenge for its enhancement under natural conditions. Recently *P. indica* association has been reported to create biotic and abiotic stress of the host plant and lead to increase in photosynthetic parameters and growth parameters. Salicylic acid has been proven to increase the plant growth and photosynthetic capacity[9]. The present comparative study was undertaken to investigate the growth parameter and photosynthetic study of *P. indica* inoculated and salicylic acid inoculated plants of *Artemisia annua.*

MATERIALS AND METHODS

(i) Plant material

Actively growing healthy fresh and uninfected young stem of *Artemisia annua* were used as explants. The plant materials were collected from a six months old *A. annua* plant growing in natural habitat of the Gauhati University Campus.

(ii) Explant preparation

Young healthy uninfected stems of *Artemisia annua*, after collection, was taken to the laboratory and immediately washed several times under running tap water. The stems were excised at the internodal portion and were soaked on liquid detergent, Tween 20 (20%) for about 10 minutes with occasional stirring followed by washing under running tap water for 15 minutes. The explants were immersed in 70 % alcohol for 1 minute and then surface sterilized with 0.1% (w/v) mercuric chloride for 0.8 seconds and subsequently rinsed thoroughly with sterile distilled water[10]. The explants were finally laid on a sterile petri plate using sterile forceps. The surface sterilized plant materials were cut into 3-4 cm in length having a diameter of 2-3mm. Thus the explants consisted of a single node containing a small portion of the internode on either side. These explants were used for shooting. For shooting the explants were inoculated in BAP. Initiation of shoots occurred within 5 days of inoculation. Microshoots were inoculated in IBA for rooting. 20 rooted plantlets were inoculated each with *P. indica* filtrate and Salicylic acid. The growth parameter and the photosynthetic pigment analysis were done. Establishment of callus culture Leaves of *Artemisia annua* were used as explants for induction of callus. Using a digital weighing scale, fresh and dry weight of the callus of *A. annua* was measured at regular intervals. The dry weight of callus was obtained by air-drying fresh callus at room temperature until a constant weight was attained. For further comparison, the callus was treated with *P. indica* filtrate and salicylic acid to study colour, texture and fresh weight and dry weight.

(iii) Shoot induction

The nodes were inoculated in MS (Murashige and Skoog, 1962) medium supplemented with 6- benylaminopurine (BAP) at different concentration (1, 2, 3 and 4 mg/l/). The effect of auxin on promotion of shoot elongation was evaluated by supplementing, naphthaleneacetic acid (NAA) at various concentration (1, 2, 3 and

4 mg/l) to BAP containing media. The explants with shoot cluster, produced after 3 weeks of culture on MS medium containing 3 mg/l BAP were transferred to fresh MS medium supplemented with varied concentration of NAA (1, 2, 3 and 4 mg/l) + BAP (1, 2, 3 and 4 mg/l) for 3 weeks to allow shoot elongation. The elongated shoots were separated and cultured for rooting on half strength MS medium and IBA as growth regulators.

(iv) Piriformspora indica

Circular agar disc (4 mm in diameter) having spores and actively growing hyphae of *P. indica*[11] were placed on 25 ml solidified Potato dextrose agar (PDA) medium in petriplates[12]. The plates were then incubated for 7 days at 30±2 °C in dark. Liquid culture made from the label-recommendation concentration (24 g/l) of potato dextrose broth which contained 4 g potato starch, 20 g dextrose dissolved in 1L of deionized water. The 14 day old *P. indica* culture was cut into 1 cm diameter and added to each flask containing sterilized PD broth. The flask is placed in an orbital shaker at 150 RPM for 15 days. After 15 days, flask content were blended with a magnetic stir bar and stirred for 2 minutes to break the fungal.

(v) Separation of culture filtrate

The fungus was filtered through four layers of sterile muslin cloth, after 15 days of inoculation and filtrate if not used was preserved for up to one month at 4 °C [13].

(vi) Preparation of elicitor

1 M salicylic acid was prepared and used as stock solution. Methanol was used as solvent to prepare the stock solution.

(v) Growth parameters of plantlets

Plant growth parameters such as root length, shoot length, dry weight and fresh weight were recorded and compared among plants containing treatment (control), treated with *P. indica* and salicylic acid.

(vi) Photosynthetic pigment analysis

We determined the chlorophyll a, b and carotenoids content of *in vitro* grown *A. annua* plantlets. Leaves were harvested, weighed, and ground in 90% ammonical acetone (acetone: water: 0.1 N ammonia, ratio of 90: 9: 1) at 4 °C. Supernatants were used to measure Chl a, Chl b, and carotenoids keeping wavelength at 663, 645, and 470 nm respectively. Total chlorophyll content was measured by UV-Vis spectrophotometer and calculated as n mol/ml[14].

Chl a = (14.21 × OD663 – 3.01 × OD645),

Chl b = (25.23 × OD645 – 5.16 × OD663), and

Carotenoids = {1000 × OD470 – (3.27 × Chl a – 1.04 × Chl b)/5}.

The obtained values were divided by leaf fresh weight to obtain values in n mol/ml/mg of leaf fresh weight.

(vii) Stastical analysis

Mean and standard deviation were calculated and Student t-test was applied to evaluate the significance of differences at $p<0.05$, wherever required [15].

RESULTS

In our study, we tested the effect of the elicitors on *in vitro* growth parameters and photosynthetic pigment of the *in vitro* grown medicinal plant *Artemisia annua*. In plant tissue culture, multiple combination of plant hormone is essential regenerate and propagate *Artemisia annua* clones for artemisinin production[16, 17]. There were many challenges in conditions for regeneration of *A. annua* regarding the use of basal medium or plant hormone based media[18]. In our investigation we have emphasized on large scale production of *Artemisia annua* within a very short time. Out of the various combinations of plant hormones used, BAP (3 mg/l) showed initiation of shooting within 5 days of inoculation in the medium. Moreover, BAP at 3 mg/l showed the highest number (60±1.5) of microshoot development from a single explant. Increased or decreased in concentration of BAP showed decreased in number of microshoot regeneration (Table 1). Of the cytokines tested, BAP is more effective in shoot induction and proliferation than kinetin. The microshoots were rooted using IBA. IBA (3 mg/l) showed initiation of shoots within a period of 12 days. Increased or decreased in concentration showed formation of callus at the basal region of the microshoot (Table 2). When the plantlets attain a height of 2 cm, treatment was done by using *P. indica* filtrate and salicylic acid. No chlamydospore can be seen at the root cortical

region of *Artemisia annua* after 10 days of cocultivation. The photosynthetic pigment analysis was regularly recorded at 5th day, 10th day and 15th day. *P. indica* treated plants showed overall increase in chlorophyll a, chlorophyll b, carotenoids (Table 3). Moreover, other growth parameters were evaluated after 1 month such as shoot length, root length, number of internodes, internode length, stem diameter, fresh weight and dry weight (Table 4). *P. indica* inoculated plants showed higher value of growth parameter followed by salicylic acid and then control. Development of callus from the leaf occurred within 15 days of inoculation. Vigorous growth of callus occurred in 2, 4 D (3mg/l) hormone concentration (Table 5) and initiation of callus occurred after 12 days of inoculation. In callus growth salicylic acid (1µl/l) showed highest fresh weight followed by *P. indica* (3µl/l) and then control (Table 6).

Table 1
Effect of BAP on induction of shoots

Hormone concentration BAP(mg/l)	% response	Average number of shoots/explants
1	42.5	15±0.9
2	50.7	25±1.8
3	96.0	60±1.5
4	66.2	45±1.9

Values within a column is the mean ±SD of three parallel experiments

Table 2
Effect of½ MS and IBA on rooting of Artemisia annua

Hormone concentration	Induction
½ MS+IBA(2mg/l)	No rooting
½ MS+IBA(2.5mg/l)	No rooting(formation of callus)
½ MS+IBA(3mg/l)	Root induction
½ MS+IBA(3.5mg/l)	No rooting (formation of callus)

Table 3
Photosynthetic pigment analysis of Artemisia annua treated with salicylic acid, P. indica and control

`Chlorophyll a			
	Control	Salicylic acid	*P. indica*
Day 05	0.35±0.01	0.45±0.35	0.57±0.01
Day 10	0.44±0.02	0.56±0.82	0.76±0.05
Day 15	0.49±0.03	0.67±0.32	0.86±0.04
Chlorophyll b			
	Control	Salicylic acid	*P. indica*
Day 05	0.14±0.01	0.17±0.10	0.20±0.02
Day 10	0.14±0.01	0.19±0.015	0.25±0.02
Day 15	0.19±0.01	0.23±0.11	0.29±0.02
Carotenoids			
	Control	Salicylic acid	*P. indica*
Day 05	34.5±0.59	39.5±0.91	47±0.69
Day 10	36.0±0.12	42.2±0.54	52.0±1.11
Day 15	37.7±1.7	47.7±1.61	58.0±0.22

Value within a column indicates mean±SD of three parallel experiments

Table 4
Growth parameters of Control, Salicylic acid and P. indica treated Artemisia annua plants

Growth parameters	Control	Salicylic acid(10µl/l)	*P. indica*(4µl/l)
Shoot length (cm)	4.0±1.1	9.8±0.44	11.5±0.33
Root length(cm)	7.1±0.23	11.2±0.34	13.8±0.11
Number of internodes	5.0±0.11	8.0±0.45	9.0±0.15
Internode length	0.3±0.29	0.7±0.78	1.1±0.32
Stem diameter (mm)	0.8±0.12	1.1±0.66	1.7±1.3
Fresh weight (gm)	0.9±0.10	1.8±0.87	2.5±1.8
Dry weight (gm)	0.4±0.43	1.3±0.32	1.9±49
Percentage colonization	-	-	90%

Value within a column indicates mean±SD of three parallel experiment

Table 5
Effect of 2,4 D and BAP on callus growth

Medium(Hormone conc.mg/l)	Colour	Texture	Growth
2,4 D(1 mg/l)	Greenish white	Friable	only induction
2,4 D(3 mg/l)	Greenish white	Friable	+++
2,4 D (6 mg/l)	Greenish white	Friable	++
2,4 D(8 mg/l)	Greenish white	Friable	++
BAP (1 mg/l)	-	-	-
BAP(3 mg/l)	-	-	-
BAP(6 mg/l)	-	-	Only induction
BAP(8 mg/l)	-	-	-

Table 6
Effect of Salicylic acid and P. indica on callus of Artemisia annua

Medium (conc. of elicitors µl/ml)		Characteristic of the callus		
		Colour	Texture	Fresh weight(g)
Salicylic acid	0.5 µl/l	Greenish white	Friable	3±0.03
	1.0 µl/l	Greenish white	Friable	4.8±0.34
	1.5 µl/l	Greenish white	Friable	4.4±0.23
	2.0 µl/l	Greenish white	Friable	2.86±0.45
	2.5 µl/l	Greenish white	Friable	2.8±1.2
	3.0 µl/l	Greenish white	Friable	2.6±0.56
P. indica	0.5 µl/l	Greenish white	Friable	1.04±0.67
	1.0 µl/l	Greenish white	Friable	1.44±0.45
	1.5 µl/l	Greenish white	Friable	2.74±0.65
	2.0 µl/l	Greenish white	Friable	2.44±0.12
	2.5 µl/l	Greenish white	Friable	2.78±1.3
	3.0 µl/l	Greenish white	Friable	4.52±1.2
Control	-	Greenish white	Friable	2.10±0.9

Value within a column indicates mean±SD of three parallel experiment

DISCUSSION

It has been reported that salicylic acid plays an important role in the growth and development of the plant[19]. Moreover, application of salicylic acid improves the photosynthetic pigment in plant[20]. Similar results have been declared by Zhao[21]and Fariduddin[22]. The beneficial effects of salicylic acid on photosynthetic rate could be attributed by its stimulatory effect on RuBiSCo activity[23]. In the present study, it was observed that salicylic acid significantly enhanced the photosynthetic pigment and biomass of *in vitro* grown *Artemisia annua* compared to control. Moreover *P. indica* inoculated plants showed significantly higher growth rate than non-inoculated plants. The growth promotional activity of *P. indica* on plants by providing essential minerals, mainly phosphate to plants[24]. The increased growth of *P. indica* colonized plants had increased growth due to an enhanced nutrient uptake (especially of phosphorus and nitrogen) from the culture medium as has been reported earlier by Shahollari[25] in cultures of Arabidopsis. It has also been revealed that *P. indica* is involved in the transportation of the phosphate to the host plant via phosphate transporter (PiPT)[26] . It is thus amply clear that a crucial determinant for the growth response of the plant is the delivery of phosphorus to the roots from the fungal hyphae [6]. Akin to mycorrhizal symbiosis[27] increase in leaf area, higher photosynthetic potential and chlorophyll levels might have resulted in increased carbon assimilation in *P. indica*-colonized plants, which could be the basis for faster development and higher biomass production[28].

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