

## Research Article



## EFFECT OF PHENYLALANINE ON QUANTITATIVE PRODUCTION OF FLAVONOIDS IN UNORGANISED TISSUE OF SOME ECONOMICALLY IMPORTANT PLANTS OF INDIAN THAR DESERT

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### ABSTRACT

Unorganized tissues of *Haloxylon recurvum*, *Haloxylon salicornicum* and *Salsola baryosma* were raised and maintained on MS medium<sup>1</sup> supplemented with 3mg/l BAP + .5 mg/l 2, 4-D, 1 mg/l BAP + 2.5 mg/l 2,4-D and 2mg/l BAP + 2mg/l 2,4-D respectively. A small amount of these tissues were transferred to fresh medium supplemented with four concentrations (20, 50, 75 and 100mg/l) of dl-β-phenylalanine (PA) and maintained for six months by frequent subculturing. The growth indices (GI) were observed in eight weeks old tissue supplemented with all concentrations of PA. Hence, this tissue was selected for analysis of flavonoids using thin layer chromatography and colorimetry. The maximum amount of kaempferol (2.62 mg/g.d.w, 2.99 mg/g.d.w and 2.57 mg/g.d.w), quercetine (2.48 mg/g.d.w, 2.82 mg/g.d.w and 2.32 mg/g.d.w) and total flavonoid contents (5.10 mg/g.d.w, 5.81 mg/g.d.w and 4.89 mg/g.d.w) was observed in the tissues of grown on standard MS medium fed with 75 mg PA/ 100ml in *H.salicornicum* and *H. recurvum* and *S. baryosma* respectively.

**Keywords:** dl-β-phenylalanine, flavonoid, growth indices, MS medium, unorganized tissues.

### INTRODUCTION

Plants of Indian arid and semi arid zones are medicinally important and good source of phytochemically important compounds. *Haloxylon salicornicum* (Moq.) Bunge in Boiss, *Haloxylon recurvum* (Moq.) Bunge ex Boiss and *Salsola baryosma* (Roem. et Schult.) Dandy known as Lana, Khar and Lani respectively are important halophytic species (Chenopodiaceae, shrub) of saline areas of The Indian Thar desert. These plants are reported to have therapeutic value in the treatment of a wide variety of disease conditions<sup>2-3</sup>. Production of valuable secondary metabolites through plant cell or organ culture is the best suited alternative to extraction of whole plant material and to increase production of secondary metabolites in *in-vitro* systems, feeding precursor or intermediate metabolites is an obvious and popular approach. Flavonoids are economically useful class of secondary metabolites. Flavonoids are a large group of polyphenolic compounds and remarkable plant metabolites that occur commonly in plants<sup>4</sup>. Cell-culture-derived flavonoids can be more easily separated in an intact polymeric form than flavonoids within complex plant tissues and overall the concentrations can be significantly enhanced in callus cultures through micro environmental control and precursor feeding to provoke metabolite production<sup>5</sup>. In order to enhance the synthesis of secondary metabolites, several organic compounds can be added to the culture medium<sup>6</sup>. The concept is based on the idea that any compound, which is an intermediate in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product<sup>7</sup>. The regulation of amino acids pathway under some stress condition dominated by the need for secondary metabolite derived from the pathway<sup>8</sup>. Flavonoids originated from phenylalanine, an upstream

metabolic precursor through phenylpropanoid pathway. Supplementation of phenylalanine is expected to increase elevated level of target compound<sup>9</sup>. In this work callus of *H. recurvum*, *H. salicornicum* and *S. baryosma* was established and grown in MS medium. The main objective of this study was to optimize precursor concentration to obtain the optimum flavonoids production of the selected plants.

### MATERIALS AND METHODS

#### Plant Material

*H. recurvum*, *H. salicornicum* and *S. baryosma* were collected from Central Arid Zone Research Institute (CAZRI), Bikaner, and surfaced disinfected using 70% of ethanol for 1 min, followed by 15% aqueous sodium hypochlorite solution for 20 min, then rinsed three times in sterile distilled water. Nodal segments of all the three plants were cut into small pieces and aseptically placed on MS medium supplemented with 3mg/l BAP + .5 mg/l 2, 4-D for *H. recurvum*, 1 mg/l BAP + 2.5 mg/l 2, 4-D for *H. salicornicum* and 2mg/l BAP + 2mg/l 2, 4-D for *S. baryosma*. Small amount of these cultured tissues were transferred to their respective MS medium supplemented with four concentrations (20, 50, 75, and 100 mg/100ml of the medium) of dl-β-phenylalanine separately. These cultures were maintained for a period of six months by frequent subculturing at interval of 6 to 8 weeks at 26 ± 10C, 55% relative humidity and diffused light conditions (3000 lux). The growth indices (GI) were calculated at different time intervals of 2,4,6,8 and 10 weeks using the formula given below.

$$\text{Growth Index (GI)} = \frac{\text{Final fresh weight of tissue} - \text{Initial fresh weight of tissue}}{\text{fresh weight of tissue}}$$



Tissue grown on MS medium supplemented with various concentrations of phenylalanine were harvested at maximum GI, dried, powdered, weighed and separately Soxhlet extracted for quantitative estimation of flavonoids. The experiment was carried out in five replicates.

#### Extraction procedure

The dried samples were separately extracted by Soxhlet method<sup>10</sup>, in 80% ethanol (100ml/g.d.w.) on a water bath for twenty four hours. Each of the extract was concentrated and re-extracted in petroleum ether (40-60°C, fraction first), ethyl ether (fraction second) and ethyl acetate (fraction third) in succession. Each step was repeated three times to ensure complete extraction in each case. Fraction first was rejected due to its richness in fatty substances, whereas fraction second was analyzed for free flavonoids and fraction third for bound flavonoids in each of the samples. Fraction third of each of the test samples was hydrolyzed by refluxing with 7% sulphuric acid (10 ml/gm residue) for two hours. The mixture was filtered and the filtrate was extracted with ethyl acetate in separating funnel. The ethyl acetate layer (upper layer) was washed with distilled water to neutrality, dried *in vacuo* and analyzed for bound flavonoids.

#### Qualitative Analysis

##### Thin Layer Chromatography

Thin glass plates (20 X 20 cm) coated (wet thickness, 0.2-0.3 mm) with silica gel 'G' (30 gm /60 ml) were dried at room temperature. The dried plates were activated at 100°C for 30 minutes in an oven and cooled at room temperature. Ethyl ether and ethyl acetate fractions from each of the test sample of all the three plants were separately applied 1 cm above the edge of the plates along with the standard reference compounds (apigenin, isorhamnetin, scutellarein, kaempferol, luteolin, quercetin, myricetin, scopoletin and negretin).

These glass plates were developed in an air tight chromatography chamber containing about 200 ml of solvent mixture of n-butanol, acetic acid and water (4:1:5, upper layer). The developed plates were air dried and visualized under UV light (254 nm) which showed one fluorescent spot in ethyl ether fraction (second) and one spot in ethyl acetate fraction (third) in all the instances, coinciding with those of the standard samples of quercetin (yellow, Rf 0.82) and kaempferol (deep yellow, Rf 0.93) in both *in vivo* and *in vitro* samples. The plates were also placed in a chamber saturated with ammonia vapors to observe the colors of the spots (quercetin–yellow, kaempferol–deep yellow). On spraying the developed plates with 5% ethanolic ferric chloride solution one spot was seen in ethyl ether fraction (second) and one spot in ethyl acetate fraction (third). Fraction second spots were coinciding with those of reference quercetin (yellowish brown) and that of fraction third with kaempferol (deep yellow) in all

samples. The Rf values were calculated as an average of the five replicates.

##### Preparative Thin Layer Chromatography (PTLC)

Glass plates (20 x 20 cm), thickly coated (wet thickness, 0.4-0.5 mm) with silica gel 'G' (45 gm / 80 ml water), activated at 100°C for 30 minutes and cooled at room temperature, were used for preparative thin layer chromatography (PTLC). The extract of both the fractions (second and third) of selected plants were applied on separate plates and developed plates were air dried and visualized under UV light (254 nm). Each of the fluorescent spot coinciding with those of the standard reference compounds of quercetin and kaempferol were marked. The marked spots were scrapped and collected separately along with the silica gel and eluted with ethanol. Each elute was then crystallized with chloroform.

The compounds thus isolated were subjected to colorimetry (for quantitative estimation), melting point (melting point apparatus, Toshniwal, India), UV maxima on a spectrophotometer (Carl-zeiss, Jena, DDR, VSU-2P) and Infra-red spectral (Perkin-Elmer, 337, Grating Infra-red spectrophotometer, using nujol or potassium bromide pellets) studies.

##### Quantitative Analysis

Spectrophotometry Quantitative estimation of the identified flavonoids was carried out colorimetrically following the method of Kariyon *et al*<sup>11</sup> and Naghski *et al*<sup>12</sup> in case of quercetin and of Mabry *et al*<sup>13</sup> in case of kaempferol. Stock solutions of quercetin and kaempferol were separately prepared by dissolving the authentic samples in methanol. Six concentrations (25 mg/ml to 150 mg/ml) of each of the standard samples were spotted on silica gel 'G' coated and activated plates. Separate plates for each of the concentrations of quercetin and kaempferol were used and these chromatograms were developed in the same solvent system as used for qualitative method (n-butanol: acetic acid: water, 4:1:5; upper layer). Such developed chromatograms were air dried and visualized under UV light (254 nm). The fluorescent spots were marked and collected along with the absorbent in separate test tubes in methanol. The mixture was shaken vigorously, centrifuged and supernatants were collected separately. The volume of elutes was made up to 10 ml by adding spectroscopic methanol. To each of these samples 3 ml of 0.1 M aluminum chloride was added, stoppered tightly and the mixture was shaken vigorously. Such tubes were kept at room temperature for 20 minutes. Five such replicates were prepared in each case and optical densities (O.D.) were measured, using spectronic 20 colorimeter (Bausch and Lomb), set at 440 nm for quercetin and at 423 nm for kaempferol against a blank (10 ml spectroscopic methanol + 3 ml of 0.1 M AlCl<sub>3</sub>). Regression curves for quercetin and kaempferol were separately plotted in between their respective concentrations and optical densities, which followed Beer's law. Each of the ethyl ether and ethyl acetate extracts was dissolved in 1 ml of



spectroscopic methanol and applied (0.1 ml) on silica gel 'G' coated plates along with authentic quercetin and kaempferol markers and developed as above. Fluorescent spots coinciding with those of the reference compounds were marked, scrapped, eluted with methanol separately and the samples were prepared as detailed above. The optical density in each case was colorimetrically recorded as above. The amount of quercetin and kaempferol in the sample were then determined (mg/100g.d.w.) by comparing with those of their respective standard regression curves. Five such replicates were examined and mean values were calculated (SD < 0.5 %).

## RESULTS AND DISCUSSION

Growth indices (GI) of unorganized tissues of *H. salicornicum*, *H. recurvum* and *S. baryosma* were calculated on standardized MS medium (MS + 2.5 mg/L 2,

4-D + 1.0 mg/L BAP, MS + 0.5 mg/L 2, 4-D + 3.0 mg/L BAP and MS + 1.0 mg/L 2, 4-D + 1.0 mg/L BAP respectively) and standardized MS medium supplemented with different concentrations of  $\beta$ -phenylalanine (25, 50, 75 and 100 mg/100ml) separately. GI of callus was found to be increased from standardized MS medium to 25, 50 and maximum (9.57 in *H. recurvum*, 8.70 in *H. salicornicum* and 9.21 in *S. baryosma*) with 75mg/100ml phenylalanine fed medium in at all growth intervals (2, 4, 6, 8, 10, and 12 weeks)<sup>14-16</sup>. GI of callus again decreased from 75 to 100 mg/100ml phenylalanine fed medium. Maximum GI was observed at the age of eight weeks old tissue fed with all concentrations of phenylalanine (table 1). Hence eight weeks old tissue supplemented with four concentrations of phenylalanine (PA) were selected for qualitative and quantitative analysis and comparison of flavonoids with that of tissues grown on standardized MS medium.

**Table 1:** Effect of  $\beta$ -phenylalanine on growth index of *H. salicornicum*, *H. recurvum* and *S. baryosma*

Plant name	Medium	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
<i>H. salicornicum</i>	ST MS MEDIUM	2.88±0.04	4.09±0.01	5.27±0.13	7.69±0.07	6.34±0.18
	MS+ 25mg PA/100ml	3.02±0.08	4.29±0.03	5.42±0.18	8.01±0.27	6.51±0.09
	MS+ 50mg PA/100ml	3.15±0.05	4.47±0.01	5.68±0.16	8.46±0.21	6.67±0.06
	MS+ 75mg PA/100ml	3.28±0.08	4.93±0.08	5.99±0.14	8.70±0.26	6.81±0.07
	MS+ 100mg PA/100ml	3.08±0.03	4.35±0.04	5.57±0.15	8.38±0.23	6.59±0.06
<i>H. recurvum</i>	ST MS MEDIUM	2.67±0.07	3.81±0.04	4.42±0.18	6.38±0.08	6.02±0.08
	MS+ 25mg PA/100ml	2.93±0.05	4.01±0.08	5.02±0.14	7.21±0.16	6.49±0.06
	MS+ 50mg PA/100ml	3.21±0.04	4.12±0.12	5.46±0.09	9.03±0.18	6.70±0.07
	MS+ 75mg PA/100ml	3.42±0.04	4.21±0.05	5.93±0.13	9.57±0.23	6.99±0.08
	MS+ 100mg PA/100ml	3.5±0.03	4.09±0.03	5.28±0.08	8.79±0.15	6.68±0.12
<i>S. baryosma</i>	ST MS MEDIUM	3.01±0.08	4.62±0.09	5.87±0.12	8.03±0.21	7.84±0.18
	MS+ 25mg PA/100ml	3.28±0.04	4.75±0.06	6.02±0.16	8.58±0.16	7.99±0.19
	MS+ 50mg PA/100ml	3.57±0.06	4.83±0.07	6.46±0.14	8.99±0.18	8.09±0.18
	MS+ 75mg PA/100ml	3.93±0.09	5.01±0.06	6.70±0.16	9.21±0.16	8.58±0.16
	MS+ 100mg PA/100ml	3.35±0.04	4.81±0.13	6.38±0.15	8.81±0.23	8.08±0.18

**Table 2:** Effect of  $\beta$ -phenylalanine on flavonoid content (mg/100 g.d.w.) in tissue cultures of *H. salicornicum*, *H. recurvum* and *S. baryosma*

MEDIA	<i>H. salicornicum</i>			<i>H. recurvum</i>			<i>S. baryosma</i>		
	Kaempferol	Quercetin	Total flavonoid	Kaempferol	Quercetin	Total flavonoid	Kaempferol	Quercetin	Total flavonoid
ST MS MEDIUM	2.38±0.12	2.12±0.04	4.50±0.08	2.57±0.06	2.44±0.04	5.01±0.13	2.31±0.06	1.96±0.04	4.2±0.18
MS+ 25mg PA/100ml	2.42±0.15	2.20±0.06	4.62±0.06	2.61±0.08	2.53±0.06	5.14±0.15	2.36±0.08	2.01±0.06	4.37±0.19
MS+ 50mg PA/100ml	2.49±0.13	2.36±0.08	4.85±0.09	2.72±0.06	2.68±0.09	5.40±0.13	2.44±0.09	2.15±0.03	4.59±0.13
MS+ 75mg PA/100ml	2.62±0.12	2.48±0.06	5.10±0.14	2.99±0.09	2.82±0.08	5.81±0.09	2.57±0.04	2.32±0.09	4.89±0.09
MS+ 100mg PA/100ml	2.40±0.09	2.16±0.12	4.56±0.13	2.60±0.04	2.47±0.06	5.07±0.14	2.34±0.03	1.99±0.04	4.27±0.12

All flavonoids individually as well as total flavonoid content showed gradual increase in amount present in tissue grown on control medium (MS) to tissues fed with 25 mg PA/100ml medium, up to maximum in tissue fed with 75 mg PA/100ml medium (as shown in Table 2). After that it started declining in tissues fed with 75 to 100 mg PA/ 100ml medium. Amount of kaempferol (2.38 mg/100g.d.w., 2.57 mg/100g.d.w. and 2.31 mg/100g.d.w.), quercetin (2.12 mg/100g.d.w., 2.44 mg/100g.d.w. and 1.96 mg/100g.d.w.) and total flavonoid

(4.50 mg/100g.d.w., 5.01 mg/100g.d.w. and 4.27 mg/100g.d.w.) in tissues of *H. salicornicum*, *H. recurvum* and *S. baryosma* respectively, grown on standardized MS medium has been estimated. A remarkable increase in the amount of flavonoid content in eight weeks old tissue grown on MS medium fed with 75 mg PA/100ml was observed in all three plant species. The maximum amount of kaempferol (2.62 mg/g.d.w, 2.99 mg/g.d.w



and 2.57 mg/g.d.w), quercetine (2.48 mg/g.d.w, 2.82 mg/g.d.w and 2.57 mg/g.d.w) and total flavonoid contents (5.10 mg/g.d.w, 5.81 mg/g.d.w and 4.89 mg/g.d.w) was observed in the tissues of grown on standard MS medium fed with 75 mg PA/ 100ml in *H.salicornicum* and *H. recurvum* and *S. baryosma* respectively. After that the amount started decreasing in tissues fed with 100 mg PA/100 ml as shown in table no. 2. Although the amount of flavonoids decreased in tissues fed with 100 mg PA/100ml even then it was sufficiently higher than amount estimated in tissues grown on standardized MS medium.

### CONCLUSION

Thus it can be concluded that tissue cultures of *H.salicornicum* and *H. recurvum* and *S. baryosma* retain the potential to synthesize flavonoids in fair amount and being one of precursors in synthesis of flavonoids, dl- $\beta$  phenylalanine fed with 75 mg PA/100ml medium can increase flavonoid content appreciably in tissue cultures.

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