

Adventitious roots formation for enhanced and sustainable production of antioxidants in *Brassica oleracea* var. *acephala* (Brassicaceae)

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Abstract: *Brassica oleracea* var. *acephala* is listed as the healthiest vegetable due to its high valued secondary metabolites content and antioxidant potential. This study was conducted to establish adventitious roots (ARs) culture as an alternative and feasible production of antioxidant secondary metabolites. ARs were induced from cotyledon explants in commercially available Murashige and Skoog (MS) plant nutrient media, gelled with 0.8% phyto-agar and supplemented with different concentration (0.1–1.5 mg·L⁻¹) of auxins (Naphthalene acetic acid; NAA, or Indole acetic acid; IAA, or Indole-3-butyric acid; IBA). AR formation responses in MS media at varying concentrations (0–50 g·L⁻¹) of sucrose and initial media pH (4, 5.0, 5.8, 7 & 8) were also studied. The bioprocessing of ARs were studied in liquid MS media containing NAA (1.5 mg·L⁻¹) as growth regulator. The growth curve, important antioxidants (phenols & flavonoids), and free radical scavenging potential of ARs were studied for a period of 9-weeks. The ARs at stationary phase (7-week) attained highest accumulation of phenols and flavonoids, which ultimately showed the highest reactive species scavenging potential. This study provides the base for production of *B. oleracea* var. *acephala* secondary metabolites on large scale to strengthen the bio-based economy of developing world.

ARTICLE HISTORY

Received: February 21, 2018

Revised: April 5, 2019

Accepted: May 30, 2019

KEYWORDS

Adventitious roots,
Phenolics,
Flavonoids,
Antioxidants,
Auxin

1. INTRODUCTION

Roots are biosynthetic factories of nutritionally and pharmaceutically important metabolites such as alkaloids, phenols, polyacetylenes, sesquiterpenes and naphthoquinones [1]. To render this potential adventitious roots (ARs) culture is the promising alternative for large scale production. It is advantageous over cell, microbial and hairy roots cultures as it has fast multiplication rate, resistant to shear-stress, genetically stable, non-GMO, and easily scalable [2]. Unlike opines, toxic chemicals production in hairy roots culture makes it ideal and acceptable to the consumers [3,4].

Brassica oleracea var. *acephala* belonging to the family *Brassicaceae* (mustard family) is economically important due to its edibility, fodder and condiment usages and oil content [5, 6]. Traditionally, this plant has been utilized as a vegetable [7, 8], ornamental plant [9] and a

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medicinal plant. Locally in Kashmir, it is treated as an important foliage herbaceous plant, is used as vegetable and taxonomically it is the oldest form of cabbage [10]. In traditional medicine the intact plant has been reported for blister formation against inflammation and warts [11] and its juices were used to relieve bronchitis, chronic cough and asthma [12]. It has also been utilized for the treatment of cardiovascular diseases and carcinomas of colon, rectum and stomach [13, 14]. These qualities are linked to its metabolites profile which varies in quality and quantity due to specie type, plant part and age, and other agronomic factors (e.g. environmental and geographic location) [15, 11]. To overcome these constraints; in-vitro cultures have been selected as an attractive, rapid and reproducible method for production of specific metabolites in bulk scale [16].

The multiple utilization practices of this plant have made it ideal for in vitro cultures establishment. In this study, we established adventitious root culture system of *Brassica oleracea* var. *acephala* and investigated the effects of different auxins, sucrose concentrations, and pH strength on ARs formation from cotyledon explants. Furthermore, the content of phenolic, flavonoids, and antioxidant potential in the bioprocessed roots were evaluated.

2. MATERIAL and METHODS

2.1. Explant source and adventitious root induction

The seeds of *Brassica oleracea* var. *acephala* were obtained from at Quaid-I-Azam University Islamabad, Pakistan. The surfaces of seeds were sterilized and then inoculated on Murashige and Skoog (1962) solid medium containing 30 g·L⁻¹ sucrose and 8 g·L⁻¹ agar [17].

For adventitious root induction, cotyledon and internode explants from 20 days old seed derived plantlet were inoculated on MS media supplemented with various concentrations (0.1, 0.5, 1.0 and 1.5 mg·L⁻¹) of Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA) and -naphthalene acetic acid (NAA) each. Additionally, the explants were cultured on MS media supplemented with varying sucrose concentrations (0, 20, 30, 40, 50, 60 and 70 g·L⁻¹) and different pH levels (4, 5.0, 5.8, 7 and 8). The data were recorded in terms of percent root induction response, number of roots induced per explant, and roots fresh and dry weight (DW) after 4 weeks of culture.

All cultures were incubated in plant growth room, where room temperature (25 ± 1°C) and humidity (70%) were kept in control. The pH of culture media was maintained at 5.8 before autoclaving. The 16h photoperiod with light intensity of 40 μmol·m⁻²·s⁻¹ was maintained in a growth room.

2.2. Submerged cultivation of adventitious roots

The 4-weeks old viable adventitious roots were aseptically excised from leaf explant and inoculated into MS medium, devoid of agar. For roots multiplication medium was supplemented with 1.0 mg·L⁻¹ NAA and 30 g·L⁻¹ sucrose. To ensure proper aeration, ~0.5 g ARs were cultured in 250 ml Erlenmeyer (conical) flask. The culture conditions in shaking incubator were set as 24h dark, 110 rpm and 25°C. The adventitious roots from shake flask were sampled after each week of culturing for a total 9-weeks of period, and biomass and secondary metabolites accumulation were measured with the time course.

2.3. Phytochemical Analysis

The increase in fresh weight (FW) and dry weight (DW), and residual media electrical conductivity (EC) were measured according to Baque et al [18]. The antioxidants, phenols and flavonoids content in ARs were measured according to Ali & Abbasi [19], and Tariq et al [20] methods. For antioxidant activity 1-diphenyl-2-picrylhydrazyl (DPPH) was used as free radical producer and its scavenging potential in ARs were measured according to Abbasi et al [21].

2.4. Statistical analysis

All experiments were carried out in triplicate and were repeated two times. Mean values of the experimented data sets were analysed for variance and significance using Duncan's Multiple Range Test (DMRT) on Statistix (8.1) software. For graphical presentation OriginPro (8.5) was used and error bars represent standard error (SE).

3.1. Adventitious root induction

Adventitious root cultures of *Brassica oleracea* var. *acephala* were established in four steps i.e. seed germination, aseptic transfer of cotyledon explant to medium, adventitious root induction and submerged cultivation in shake flasks (Fig 1).

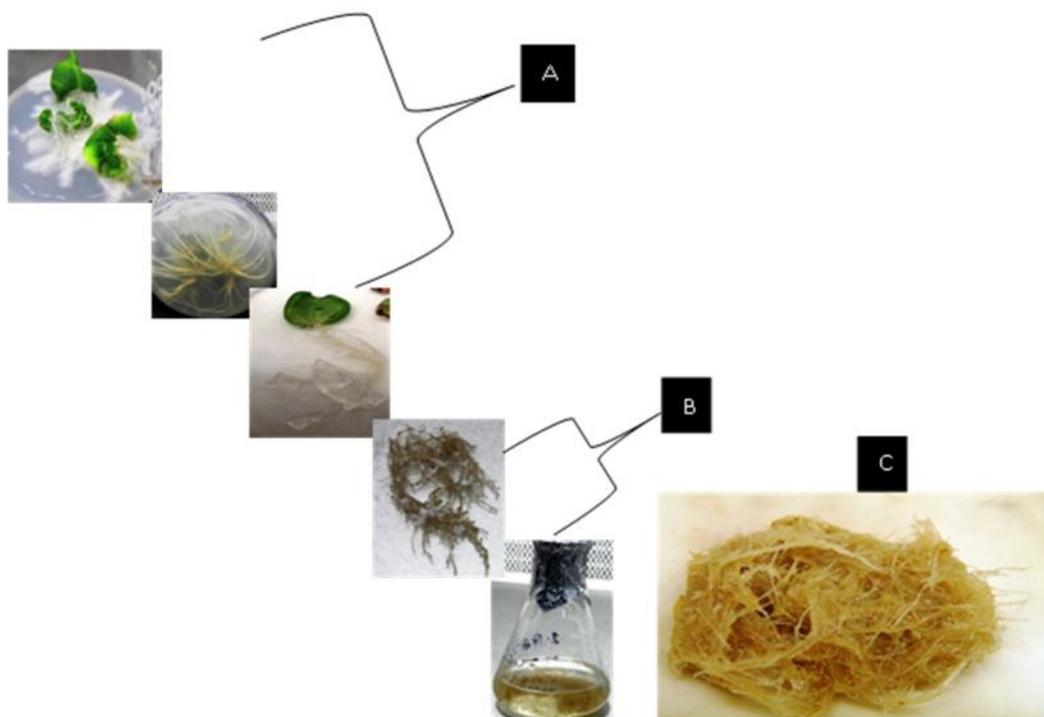


Figure 1. Adventitious root culture establishment **A)** Induction and elongation of adventitious roots **B)** Submerged cultivation, and **C)** Fresh Biomass.

Cotyledon explants cultured on to MS basal media supplemented with various amounts ($0.1, 0.5, 1.0$ and $1.5 \text{ mg}\cdot\text{L}^{-1}$) of auxins; NAA, IBA or IAA failed to produce shoots and resulted in induction of the primary adventitious roots at the cut ends in varying frequency (Table 1). However, internode explants induced shorter adventitious roots with maximum frequency (34%) in response to $1.0 \text{ mg}\cdot\text{L}^{-1}$ NAA. The cotyledon explants inoculated on MS medium containing $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA resulted into highest adventitious root induction frequency (87%) and maximum number of roots per explant (35), fresh weight (3.0 g) and dry weight (0.26 g). In response to $1.5 \text{ mg}\cdot\text{L}^{-1}$ of IAA, maximum (70.7%) adventitious roots induction was observed. Different concentrations of IBA resulted into significantly lower biomass accumulation and rooting induction frequency.

Table 1. Auxin type and concentration effect adventitious root formation in cotyledon explant of *B. oleracea* var. *acephala*.

Treatment (mg l ⁻¹)	Rooting frequency (%)	Number of roots	Fresh Weight (g/culture)	Dry Weight (g/culture)
MS ⁰	ND*	ND	ND	ND
NAA 0.1	68.7 ± 2.96bc*	14.3 ± 1.97c	1.96 ± 0.034c	0.150 ± 0.017abc
0.5	87.3 ± 1.45a	35.06 ± 2.31a	2.96 ± 0.035a	0.232 ± 0.088a
1.0	79.0 ± 1.53ab	24.33 ± 2.62b	2.76 ± 0.038a	0.239 ± 0.007a
1.5	76.7 ± 2.60ab	26.90 ± 1.07ab	2.36 ± 0.037b	0.199 ± 0.006ab
IAA 0.1	ND	ND	ND	ND
0.5	20.7 ± 2.33d	2.00 ± 1.15d	1.09 ± 0.042d	0.088 ± 0.003abc
1.0	32.7 ± 2.03d	4.53 ± 1.47d	1.15 ± 0.052d	0.094 ± 0.004bc
1.5	70.7 ± 1.76b	7.33 ± 1.05d	1.29 ± 0.058d	0.136 ± 0.011abc
IBA 0.1	ND	ND	ND	ND
0.5	27.30 ± 2.34d	1.60 ± 0.92d	1.05 ± 0.028d	0.063 ± 0.005bc
1.0	31.23 ± 2.69d	1.46 ± 0.41d	1.053 ± 0.030d	0.086 ± 0.008abc
1.5	56.50 ± 2.18c	3.59 ± 1.30d	1.18 ± 0.056d	0.010 ± 0.002bc

Data values represent mean ± SE of three replicates.

*different alphabets in columns notes the significant difference at P<0.01

*ND: Not Detected

Significant variation in biomass accumulation was observed in response to different concentrations of sucrose while keeping the NAA concentration to 1.0 mg·L⁻¹. MS medium supplemented with 40 g·L⁻¹ sucrose was found to display maximum values for adventitious root induction frequency (86%), number of roots per explant (16.2) and fresh weight (2.3 g/culture) and dry weight (0.12 g/culture) (Table 2). Further increase in sucrose concentration beyond 40 g·L⁻¹ significantly reduced adventitious root induction frequency.

Table 2. The effects of different sucrose concentrations in MS medium on induction of adventitious roots from cotyledon explant.

Sucrose Concentration (g L ⁻¹)	Rooting frequency (%)	Number of roots	Fresh weight (g/culture)	Dry weight (g/culture)
0	ND	ND	ND	ND
20	40.64 ± 2.4c	3.33 ± 1.3c	0.81 ± 0.1b	0.05 ± 0.012a
30	59.90 ± 1.3b	7.33 ± 1.9bc	1.73 ± 0.4ab	0.09 ± 0.023a
40	86.67 ± 1.5a	16.30 ± 1.5a	2.29 ± 0.2a	0.12 ± 0.034a
50	77.80 ± 2.7a	10.96 ± 2.5ab	1.42 ± 0.2ab	0.06 ± 0.025a
60	51.15 ± 2.3bc	4.59 ± 0.9c	0.70 ± 0.3b	0.04 ± 0.003a
70	25.74 ± 1.9d	2.67 ± 1.8c	0.50 ± 0.1b	0.03 ± 0.007a

Data values represent mean ± SE of triplicates.

*ND; not detected

*values annotated with different alphabets are significant at P<0.01

Among different levels of pH tested, while keeping sucrose 30 gL⁻¹ and NAA 0.5 mgL⁻¹ constant, highest adventitious root induction frequency (84.6%) with maximum number of roots per explant (21.04) and fresh weight (2.5 g/culture) and 0.24 g dry weight (0.24 g/culture) were recorded in response to pH 5.8. Significant decrease in adventitious rooting was observed at all other pH levels compared to 5.8 (Table 3).

Table 3. The effects of hydrogen ion concentration (pH) on formation of adventitious roots from cotyledon explant.

Hydrogen ion concentration (pH)	Rooting Frequency (%)	Number of Roots	Fresh Weight (g/culture)	Dry Weight (g/culture)
4	52.21 ± 2.78c	8.25 ± 2.03bc	0.98 ± 0.13b	0.07 ± 0.01a
5	70.66 ± 1.503b	10.05 ± 2.07b	1.19 ± 0.11ab	0.18 ± 0.08a
5.8	84.63 ± 2.04a	21.04 ± 2.77a	2.50 ± 0.29a	0.24 ± 0.07a
7	47.49 ± 2.09c	5.28 ± 1.79bc	0.82 ± 0.35b	0.06 ± 0.02a
8	22.04 ± 2.65d	1.81 ± 1.06c	0.45 ± 0.26b	0.02 ± 0.004a

*values are means ± standard error of three replicates and different alphabets denotes significance at P<0.01

3.2. Adventitious root culture

Biomass formation of the adventitious root culture in suspension culture of *Brassica olerace* var. *acephala* showed a swift inclined in growth curve. This inclined was characterized by an initial lag phase of 7 days for fresh and dry weight, followed by log phase of 35 days and a subsequent stationary phase during 63 days period of culture (Fig 2). Maximum fresh weight (FW) and dry weight (DW) of 3.19±0.044 and 0.338±0.006 g/culture, respectively, were observed on the 49th day of culture. A highest fresh weight (FW), ~6-times than the initial inoculum weight (0.5 g) of ARs were attained in shake flask bioreactor. This increase in roots FW was also characterized by steady decrease in culture volume with time, and was linked with the nutrients and water ingestion by roots for growth and biomass accumulation.

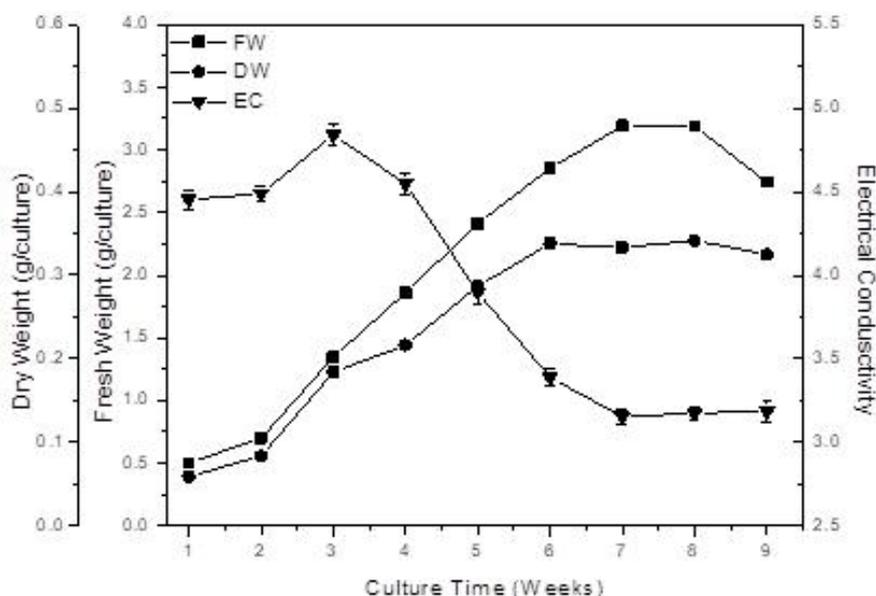


Figure 3. Total phenolic content (TPC) and total flavonoid content (TFC) with respect to dry weight (DW) accumulation.

The electrical conductivity (EC) of medium showed a gradual decline which can be linked with the nutrients consumption by the growing roots. This decrease was characterized by an initially increase and might be explained from an assumption that roots excrete metabolites for its adjustment to the new environment. The increase in biomass accumulation and decline in EC of exhausted MS media are associated with the nutrients uptake (PO_4^- , NH_3^+ , NO_3^- , etc.) from media by the ARs [24].

In the present study, the overall pattern of total phenolic content and total flavonoid content accumulation in adventitious root cultures displayed a growth-dependent pattern. TPC and TFC detected in 1 week old cultures were $3.39 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$ and $0.24 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$, respectively, that reached to its respective maximum values of $27.4 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$ and $8.8 \text{ mg}\cdot\text{g}^{-1} \text{ DW}$ in 7-week-old cultures (Fig 3). The phenolic and flavonoid content in ARs were termed as gallic acid and quercetin equivalent, respectively based on the used standers (gallic acid and quercetin).

3.3. Antioxidant activity

Antioxidant activity of adventitious root cultures was determined by three different methods; DPPH radical scavenging assay, reducing power assay and total antioxidant capacity (Fig 4). These activities were estimated as ascorbic acid equivalent (AAE). Highest levels of DPPH radical scavenging activity ($73.1\pm 5.9\%$), reducing power ($1.51\pm 0.17 \text{ mg AAE/g DW}$) and total antioxidant activity ($3.66 \pm 0.13 \text{ mg AAE/g DW}$) were displayed by 7-week old cultures (Fig 4).

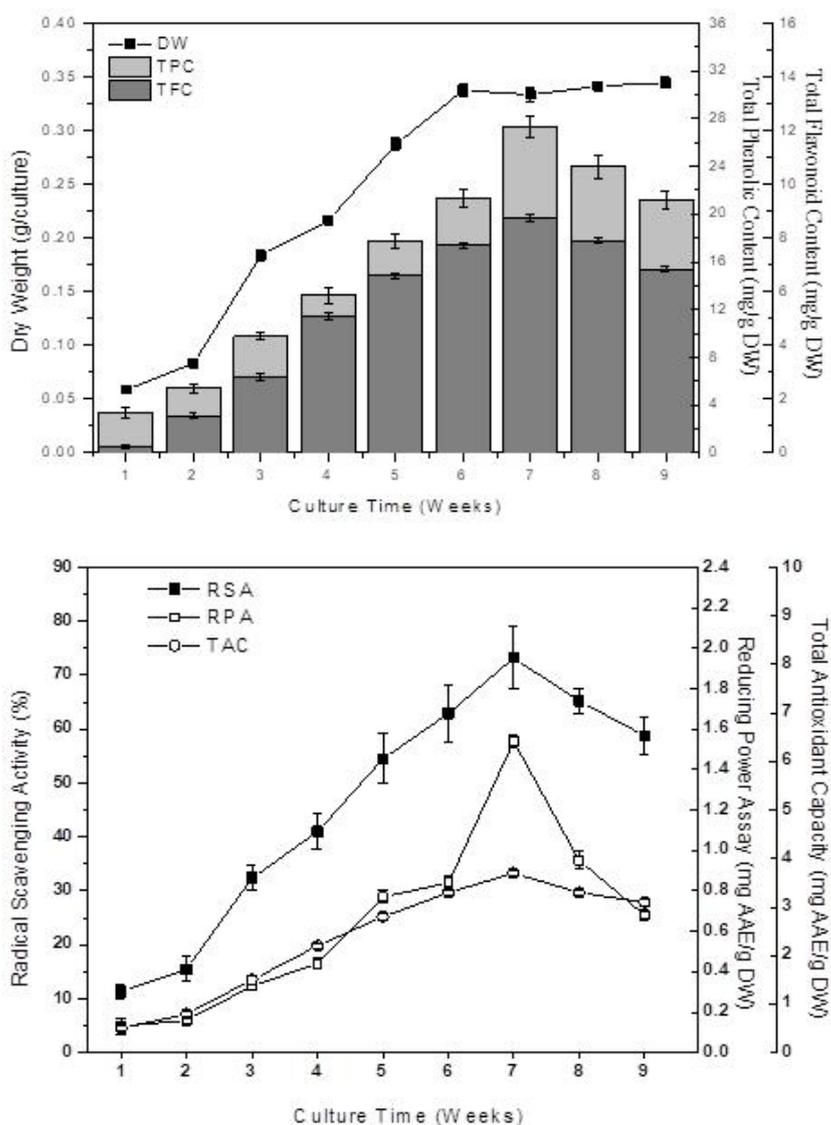


Figure 4. Antioxidant activities of adventitious root cultures as radical scavenging activity (RSA), reducing power assay (RPA) and total antioxidant capacity (TAC), with respect to time of harvest.

4. DISCUSSION

In the preliminary studies, internode and cotyledon explants were cultured for adventitious root induction. Among both the explants investigated, maximum 87% adventitious root formation was found in cotyledon explants; however internodes explants remained quiescent during the same culture conditions and minimum 34% rooting was observed. The morphological and physiological differences in these two explants explains the different rooting tendency at the same hormonal treatments [25]. By using cotyledon explants in the subsequent experiments, the adventitious root induction frequency was more pronounced with NAA compared to similar concentrations of IAA and IBA. These ARs in NAA containing MS medium were many in number, thick and white in appearance, and shorter in length without lateral branching. However, ARs in IAA supplemented media were few in number, profusely branched and slender in strength.

We found that sucrose concentration and pH level effected adventitious root induction significantly. MS medium augmented with 40 g·L⁻¹ of sucrose was supportive for maximum percent root induction while sucrose level higher than 40 significantly impaired the roots formation in cotyledon explants. As has been reported, sucrose act as building block of living cell [26] and adjust the cellular osmotic potential [27], the decrease in biomass accumulation at elevated sucrose concentration might be attributed to higher osmotic pressure that is deleterious to root primordia growth [28]. Similar observations were also made by Baque et al. [4] and Wang & Weathers [28].

Medium pH is reported to effect the solubility of nutrient elements by changing their ionic forms [24]. The decrease in biomass accumulation at both extremes of pH (4.0 and 8.0) might be due to the availability of some nutrient elements, like trace elements at acidic pH are more available while calcium (Ca) and phosphorous (P) are less available [29].

Plants are the source of structurally diverse secondary metabolites which are grouped into several classes (phenols, alkaloids, saponins, etc) and among these polyphenols are considered as the largest class of organic antioxidants [30]. These phenolics antioxidants are safer than vitamin C and E supplements, and more potent to scavenge the reactive free radicals [31]. The present study reports the viable alternative for large scale production of antioxidants in ARs culture, which can be scaled up to bioreactor scale for commercial production. We found a positive correlation of phenolics and flavonoids with antioxidant activities in adventitious root cultures. Several studies attributed the antioxidant activity (DPPH, total antioxidant activity and reducing power) to phenolic content in plant samples [32, 33], which have been proved to be more potent than synthetic antioxidants. Earlier authors [34, 35] have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. Previously, adventitious root cultures for other medicinal plants have been reported but this study reports ARs culture of *Brassica oleracea* var. *acephala* for the first time to produce phenolic and flavonoids using shake flask bioreactor [4, 27, 36, 37]. The fast growth rate of ARs in this study also opens the gate to genetic engineers for recombinant therapeutic proteins production.

5. CONCLUSION

The present study showed growth-associated increase in total phenolic content and total flavonoid content, which were found in a linear correlation with antioxidant activities. Additionally, this protocol can be exploited for production of other medicinally valuable secondary metabolites including glucosinolates and brassinosteroids.

Acknowledgements

We do acknowledge the Quaid I Azam University Islamabad, Pakistan for providing the resources and consumables to conclude this work. Also we extend our thanks to the Higher Education Commission (HEC), Pakistan for facilitating the research culture and promoting the higher education in the country.

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