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A STUDY ON THE USE OF A-AMYLASE INHIBITOR FROM *VIGNA UNGUICULATA* SEEDS AS AN ORGANIC NITROGEN SOURCE IN PLANT TISSUE CULTURE

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ABSTRACT

α-Amylase inhibitor from *Vigna unguiculata* seeds (VUAI) was purified using ion-exchange chromatography on Poros HS-50 column. Plant cell culture was used as it provides a controlled environmental and nutritional condition. VUAI used in different concentrations as 10, 100 and 200 ppm was found to increase the biomass of both grape and peanut callus cells in liquid media. Increased anthocyanin content in grape callus and total phenolics content in peanut callus was observed in VUAI treated groups. The VUAI treated groups showed better antioxidant activities than the control group. The SDS-PAGE profile showed a marked increase in the number of secreted proteins in the VUAI treated groups.

KEYWORDS: **Anthocyanin, *Arachis hypogaea,* biomass, polyphenols, *Vitis vinifera***

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INTRODUCTION

Vigna unguiculata (Cowpea) is an important legume that can be easily cultivated in areas of low rainfall and poor soil [1]. It is one of the most important source of proteins, carbohydrates, vitamins, antioxidants and trace elements in the developing countries [2,3]. *Vitis vinifera* (Grape) is one of the most important fruit crops and it is extensively grown around the world. Colour plays an important role in the organoleptic qualities and market value of grapes. This colour depends on the composition and content of anthocyanins [4]. Anthocyanins have beneficial health effects due to their antioxidant activities, hence have applications as anti-cancer agents and are effective against cardiovascular disorders [5]. Apart from these health benefits, anthocyanin pigments have great demand in the commercial market as they can be used as natural dyes in the food and textile industries [6]. *Arachis hypogaea* (Peanut) is one of the most important oilseed crops in the semiarid and tropical regions of the world. It is the second most harvested legume in the world. The health benefits of peanuts have been attributed to the presence of phenolics compounds which have a potential role as antioxidative and anti-inflammatory agent [7-9]. Enzyme inhibitors from crop seeds are important not only because of their defensive role but also due to their activity as storage proteins [10]. Successful generation of plant cell culture biomass *in vitro* for production of medicinal compounds is of utmost importance [11]. Plant cell cultures can be beneficial if the compound of interest can be produced in a short period of time, utilizing a very small amount of plant material. It also has the added advantage of controlled environmental and nutritional conditions that ensures a continuous yield of metabolites [12]. Differentiation of the callus cells is controlled by various growth regulators in the culture medium [13]. In view of these objectives, the aim of the present study was to evaluate a possible use of proteinaceous α-amylase inhibitor from *Vigna unguiculata* seeds (VUAI) on the growth and secondary metabolite production in grape and peanut callus cultures.

MATERIALS AND METHODS

Seeds of *Vigna unguiculata* and *Arachis hypogaea* (Peanut cultivar SB-11) were purchased from the local market. Previously developed and proliferated callus culture of *Vitis vinifera* var. Thompson seedless grape available at Plant Tissue Culture Division, National Chemical Laboratory were used for testing of effect of VUAI. All chemicals and reagents used were of analytical grade.

(i) Peanut callus induction

Seeds were surface sterilized using 1% bavistin and 4% savlon (v/v). The seeds were then disinfected using 0.1% mercuric chloride (w/v). The adhering mercuric chloride was removed by rinsing the seeds with sterile deionized water. The testa of these seeds was removed aseptically, and the seeds were cultured on Whatman filter paper support in deionized water in test tubes. The pH of the deionized water was adjusted to 5.8 prior to autoclaving. After incubating the cultures for 3-4 days in dark for radical emergence, the seedlings were transferred to 16 h photoperiod of 32 µmol photons m^{-2} s^{-1} at 25 ± 2°C. After a period of 15 days, leaves of well grown peanut seedlings were taken as explant for callus induction on basal media containing Murashige and Skoog (MS) salts and B5 vitamins. The media was supplemented with N^6-benzylaminopurine (BAP 1ppm), α-naphthalene acetic acid (NAA 1ppm) and 3% sucrose [14].

(ii) Isolation and purification of α-amylase inhibitor

The *V. unguiculata* α-amylase inhibitor (VUAI) was isolated from a saline extract (100 g seeds in 500 ml 0.85% NaCl) and purified by ion exchange chromatography on a Poros HS 50 column using a discontinuous salt gradient (0.1, 0.2, 0.5 and 1.0 M NaCl) in acetate buffer (pH 5.4, 10 mM; buffer A) for elution. Each

fraction was monitored for α-amylase inhibitory activity. The α-amylase inhibitor rich fractions were pooled, lyophilized and used for further studies.

(iii) Effect of VUAI on callus in liquid media

The experiment was conducted in triplicates in side arm flasks. VUAI was sterilized by passing it through 0.2 µm membrane filter. It was then added at a concentration of 10, 100 and 200 ppm respectively to the 20 ml liquid media in side arm flasks containing 500 mg of one month old callus of grape or peanut. For grape callus, MS basal medium supplemented with 1ppm of BAP, 0.1ppm of NAA and 2% sucrose was used. The flasks were kept on shaker at 90 rpm in dark for 10 days for grape and 14 days for peanut for the multiplication of cells. Simultaneously, controls without VUAI were also kept. The cell multiplication was estimated by taking the absorbance at 660 nm on a colorimeter (Kanad Vidyut, H0392, India). Similar experiment was performed using Casein Hydrolysate at similar concentrations of VUAI used to compare the difference in the growth pattern with respect to the VUAI test group.

(iv) Protein profiling

At the end of 10 days for grape callus and 14 days for peanut callus, the liquid cell suspension media was centrifuged at 10,000 rpm for 15 min at 4°C and the fresh cell biomass was determined, while the supernatant was used to profile the presence of extracellular proteins using SDS-PAGE.

(v) Isolation of α-amylase

Extraction of α-amylase from grape and peanut callus was done to check the effect of VUAI on the same. Extraction was performed according to the procedure described by Kim et al [15] with slight modification. 0.5 g of callus (fresh weight) was homogenized with 2 ml of buffer A (containing 2 mM $CaCl_2$) followed by centrifugation at 8,000 rpm for 15 min. The supernatant thus obtained was dialyzed against buffer A and assayed for α-amylase activity.

(vi) α-amylase assay

The α-amylase assay was performed in triplicates according to the procedure described by Kumar et al [16] with slight modification as follows; 0.1 unit of the α-amylase was incubated with 100 µl of 2% (w/v) starch azure (Sigma biochemicals, USA) solution at 37°C for 15 min in buffer A. Then the reaction was terminated by the addition of 0.5 ml of 50% acetic acid solution, followed by centrifugation at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 600 nm against the corresponding control. One unit of α-amylase activity liberates the chromolytic product to the final absorbance A_{600} = 1.

(vii) α-amylase inhibitor assay [16]

The assay was performed essentially as described above except that 0.1 unit of the α-amylase was pre-incubated with 0.2 ml (65 µg) of VUAI at 37°C for 15 min in buffer A after which the reaction was initiated by addition of starch azure. Corresponding controls were run simultaneously. The inhibition was expressed as residual α-amylase activity (%) compared with the uninhibited sample.

(viii) Determination of anthocyanin concentration in grape callus cells

Anthocyanins were extracted from the grape callus cells according to the procedure described by Hiratsuka et al [17] with slight modification. 200 mg of grape callus cells were taken and sonicated in 2 ml of methanol containing 1% HCl at 4°C for 20 min. The extract was then kept overnight in dark at 4°C followed by centrifugation at 12,000 g for 15 min. The supernatant thus obtained was used as a source of anthocyanin.

The concentration of anthocyanin was determined by the pH differential method as described by Lee et al [18] and the concentration of anthocyanin was calculated by using the formula as follows,

Anthocyanin pigment (cyanidin-3-glucoside equivalents, mg/L)

$$= \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

Where
$A = (A_{520nm} - A_{700nm})$ pH 1.0 – $(A_{520nm} - A_{700nm})$ pH 4.5,
MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu),
DF = dilution factor, l = pathlength in cm,
ε = 26,900 molar extinction coefficient, in L x mol^{-1} x cm^{-1} for cyd-3-glu, and
10^3 = factor for conversion from g to mg

(ix) Total Phenolics Contents (TPC) Determination from peanut callus

The phenolics were extracted by homogenizing the peanut callus (0.5 g) with 5 ml of methanol at 4°C. The homogenate was centrifuged and the supernatant was used a source of total phenolic compounds. TPC was determined by Folin-Ciocalteu procedure using gallic acid as a standard as described by Mar Mar Win et al [8]. The results obtained were expressed as mg gallic acid equivalent (GAE)/g sample. All the measurements were performed at 750 nm and the tests were carried out in triplicates.

(x) Radical scavenging assay

The antioxidant capacity of anthocyanin and total phenolic extract was estimated by 2, 2-diphenyl-1-1 picrylhydrazyl (DPPH) assay as described by Mar Mar Win et al [8] with the only difference of using ascorbic acid as a standard. DPPH in methanol was used as a control. All tests were carried out in triplicates. The antioxidant capacity was expressed as percent DPPH scavenged by each sample and calculated by the following formula,

$$\% \text{ DPPH scavenged} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

(xi) Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was carried out by One way ANOVA followed by *post hoc* Dunnett's multiple comparison test, performed using GraphPad InStat version 3.00 for Windows Vista™ BASIC, GraphPad Software, San Diego, CA. P <0.05 was considered statistically significant

RESULTS

Growth pattern of grape and peanut callus cells in presence of VUAI and Casein Hydrolysate in liquid media

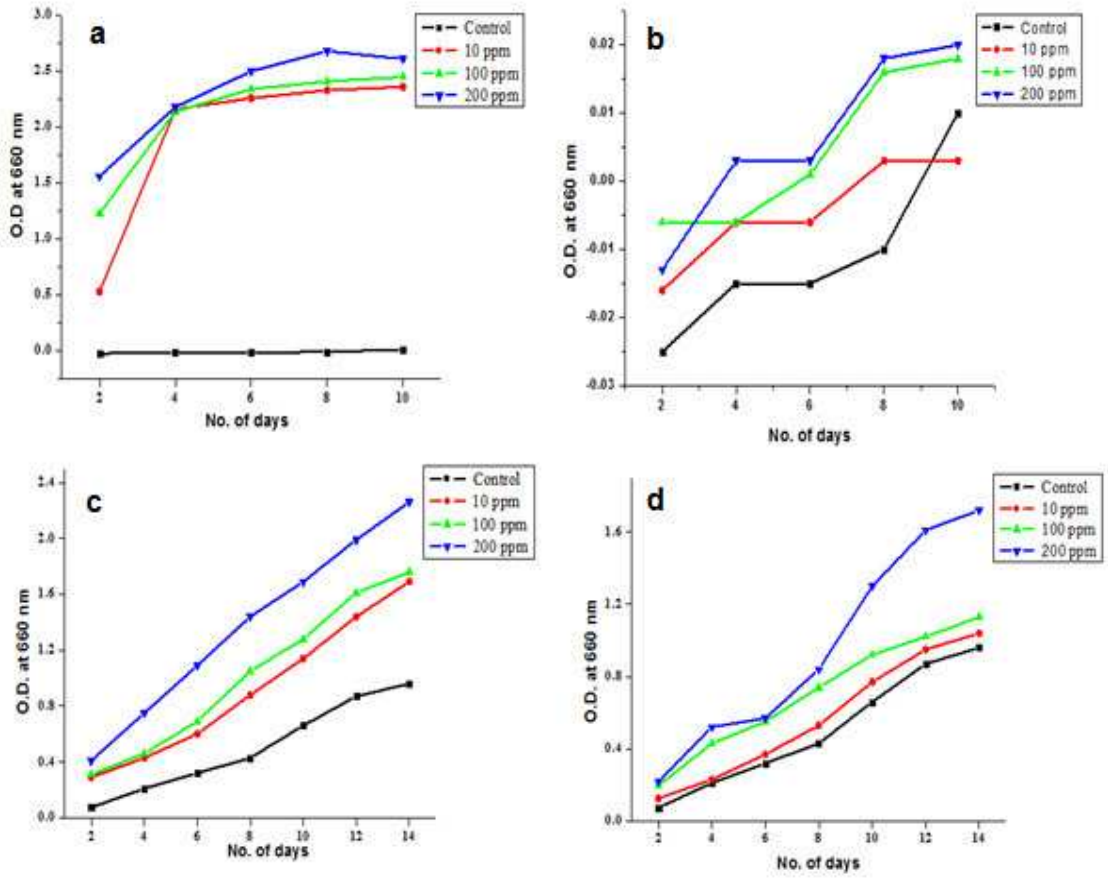

Figure 1

a) Grape callus cells in presence of VUAI b) Grape callus cells in presence of Casein Hydrolysate c) Peanut callus cells in presence of VUAI d) Peanut callus cells in presence of Casein Hydrolysate

The time taken for the VUAI treated groups to show maximum increase in cell biomass in both grape and peanut callus (Fig. 1a, 1c) was much less with respect to the control and the Casein treated groups (Fig. 1b, 1d). Similarly an increase in the total cell number and hence in the biomass (Table 1) was seen in the VUAI treated groups with respect to the Casein treated groups (Fig. 1, 2). An increase in the anthocyanin content in the VUAI treated grape callus cells with respect to the Casein treated group was observed (Fig. 2a, 2b).

Difference in the cell biomass of grape and peanut callus cells in presence of VUAI and Casein Hydrolysate in liquid media

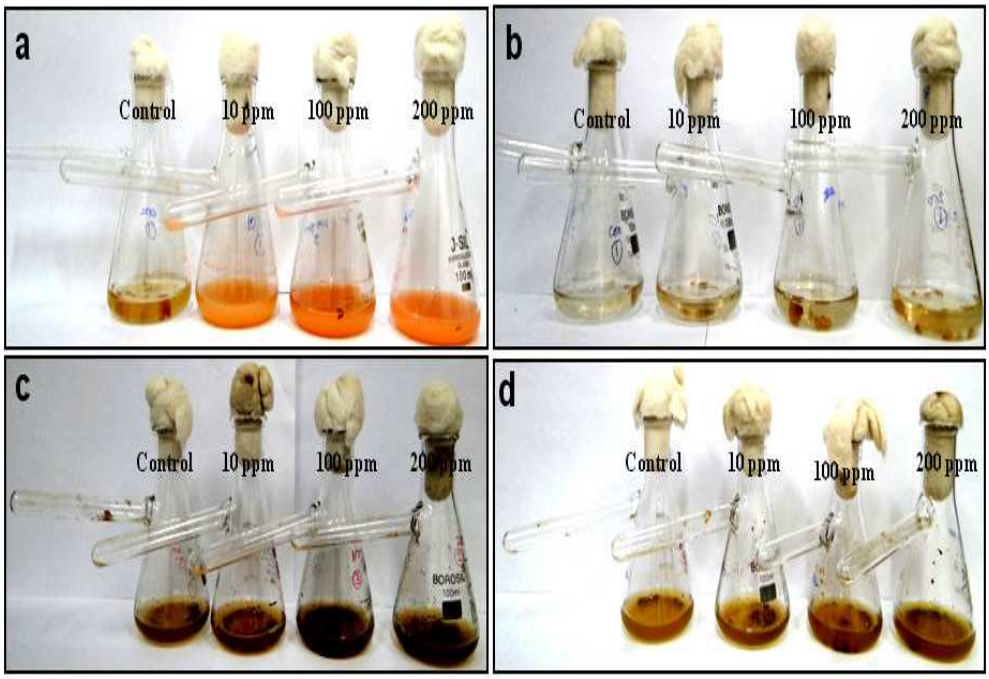

Figure 2
a) Grape callus cells in presence of VUAI b) Grape callus cells in presence of Casein Hydrolysate c) Peanut callus cells in presence of VUAI d) Peanut callus cells in presence of Casein Hydrolysate

A decrease in the amylase activity was observed (Table 2) in the VUAI treated groups as compared to the control group. As the VUAI concentration was increased from 10 ppm to 200 ppm, a linear increase in the anthocyanin and total phenolic content was observed in peanut callus (Table 2).

Table 1
Effect of VUAI on the biomass of grape and peanut callus cells†

Parameters	Control	10 ppm	100 ppm	200 ppm
Fresh weight (g) (Grape + VUAI)	0.58 ± 0.039	0.83 ± 0.05^{ns}	1.0 ± 0.08*	1.53 ± 0.25**
Fresh weight (g) (Grape + casein)	0.56 ± 0.04	0.63 ± 0.045^{ns}	0.68 ± 0.04^{ns}	0.77 ± 0.07*
Fresh weight (g) (Peanut + VUAI)	0.75 ± 0.043	0.91 ± 0.055 ^{ns}	1.59 ± 0.24*	2.24 ± 0.32**
Fresh weight (g) (Peanut + casein)	0.74 ± 0.06	0.83 ± 0.052 ^{ns}	1.03 ± 0.15 ^{ns}	1.17 ± 0.21 ^{ns}

†Data were expressed as mean ± SEM. P<0.05 was considered significant. *P<0.05, **P<0.01 and P<0.001***

An increase in the number of cells accumulating anthocyanin (Fig.1) with respect to the control and Casein Hydrolysate treated groups was observed. Both the anthocyanin and the phenolic compounds extracted from the VUAI treated groups showed higher DPPH scavenging activity with

respect to the Casein Hydrolysate treated and control groups. This increase was observed to be linear with the increase in the concentration of VUAI (Table 2).

Table 2
Effect of VUAI on the antioxidant and α-amylase activity of grape and peanut callus cells‡

Parameters	Control	10 ppm	100 ppm	200 ppm
cyd-3-glu equivalent mg/L (Grape + VUAI)	8.21 ± 0.1	17.54 ± 0.28**	22.83 ± 0.34**	47.54 ± 0.23**
cyd-3-glu equivalent mg/L (Grape + casein)	8.21 ± 0.18	8.8 ±0.36 ns	9.5 ± 0.39**	9.86 ± 0.56**
mg GAE/g of sample (Peanut + VUAI)	10.15 ± 0.22	12.06 ± 0.93ns	17.09 ± 2.00**	20.51 ± 0.78**
mg GAE/g of sample (Peanut + casein)	10.03 ± 0.64	10.73 ± 1.1ns	15.33 ± 1.79*	17.8 ± 0.5**
% DPPH scavenged (Grape + VUAI)	11.66 ± 1.52	20.55 ± 1.06**	32.4 ± 1.75**	41.1 ± 1.51**
% DPPH scavenged (Grape + casein)	11.1 ± 1.5	12.5 ± 0.9 ns	15.5 ± 0.6*	16.36 ± 0.5*
% DPPH scavenged (Peanut + VUAI)	13.56 ± 0.75	19.73 ± 0.55*	28.0 ± 1.08**	38.23 ± 1.91**
% DPPH scavenged (Peanut + casein)	13.066 ± 1.70	16.56 ± 0.86 ns	19.73 ± 0.47*	21.03 ± 1.48**
Units of α-amylase (Grape + VUAI)	0.35 ± 0.02	0.246 ± 0.05ns	0.196 ± 0.04*	0.123 ± 0.03**
Units of α-amylase (Peanut + VUAI)	0.29 ± 0.04	0.211 ± 0.034ns	0.185 ± 0.02 ns	0.129 ± 0.01**

‡Data were expressed as mean ± SEM. P<0.05 was considered significant. *P<0.05, **P<0.01 and P<0.001***

The SDS-PAGE profile of the extracellular proteins (Fig. 3) showed that there was a marked increase in the number of secreted proteins in the VUAI treated groups with respect to the control and Casein Hydrolysate treated groups.

SDS-PAGE profile of extracellular proteins

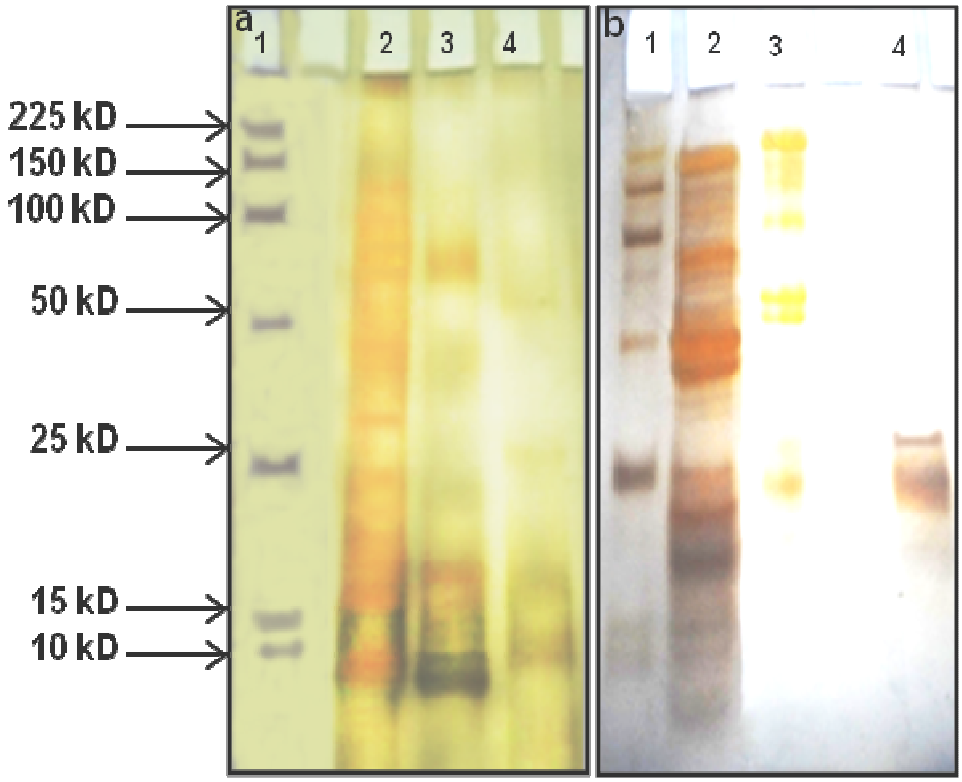

Figure 3

a) From grape callus cells b) From peanut callus cells Lane 1: Molecular weight marker; Lane 2: Supernatant from grape/peanut callus cells treated with 200 ppm of VUAI; Lane 3: Supernatant from grape/peanut callus cells treated with 200 ppm of Casein Hydrolysate; Lane 4: Supernatant from grape/peanut callus cells (control)

DISCUSSION

Cowpea is a drought resistant crop. It is a good and cheap source of protein in developing countries [19]. There are reports of presence of enzyme inhibitors such as α-amylase inhibitors in cowpea [20]. These α-amylase inhibitors can act as storage proteins or N-reserve proteins as they have specific amino acid composition and therefore help to compensate in part for the deficiency of essential amino acids [21]. Plant cell cultures can be easily manipulated and hence are highly important for the large scale production of industrially important and therapeutic proteins [22]. Liquid media provides a uniform culturing condition that can be used efficiently not only for the mass propagation *in vitro* but also for secondary metabolite production [23]. Grapes and peanuts are important dietary sources of antioxidants and it would be useful to improve the yield of these antioxidants as they have beneficial effects on human health [24]. Hence the effect of VUAI on the production of anthocyanins and overall phenolic compounds was checked in liquid media using grape and peanut callus cultures for the same. Casein Hydrolysate is a commercially available protein supplement routinely used in the plant tissue culture studies [25]. Therefore, it was used as a standard reference to compare the results obtained with VUAI. The continued increase in culture biomass is due to an increase in cell number of productive cells [26]. The increase in the biomass (Table 2) of the VUAI treated groups may be possibly due to this increase in the number of cells. The α-amylase inhibitors from plant sources do not

inhibit the plant α-amylases but can inhibit mammalian and insect α-amylases depending upon their specificity of recognition [27]. Organic nitrogen sources tend to decrease the α-amylase production in the media [28]. Since VUAI was used as an organic nitrogen source, it explains the observed decrease in the level of α-amylase production (Table 2) in the VUAI treated groups as compared to the control groups. Our results are well in agreement with the observations of Tholakalabavi et al [29] that organic nitrogen source tends to increase the pigmented cell content of grape callus cultures. Supplementing the media with certain additives tends to increase the secretion and accumulation of extracellular proteins in the culture media, which have the added advantage of easy product recovery and purification [22]. The SDS-PAGE profiles thus obtained support the possible role of VUAI in increasing the expression of extracellular proteins (nature of the proteins not determined) in the media by both grape and peanut callus cells. The work described in this paper is useful in many ways; (a) The commercial use of plant tissue culture should have minimum input expenses which can be achieved by substituting the expensive chemical nutrients with low cost natural extracts [25]. VUAI offers a good low cost natural additive for plant tissue culture for increasing the cell biomass in a short period of time. (b) Plants usually produce small amounts of pharmacologically important secondary compounds but industries require large scale production of such metabolites [12]. Addition of VUAI enhanced the production of important metabolites such as anthocyanins and phenolic compounds without any seasonal constraints. (c) The seed meal obtained after removal of VUAI from the cowpea seeds can be used as a good proteinaceous feed for animals.

CONCLUSION

Our results suggests that VUAI may offer a good organic nitrogen source to be used in plant tissue culture studies, as it was found to enhance not only the biomass production but also the content of phenolic compounds, which have several health benefits.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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