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EFFECT OF 2,4-D ON PHENOLICS PRODUCTION AND DETECTION OF *IN-VITRO* CULTURE-INDUCED VARIATION THROUGH INTER-SIMPLE SEQUENCE REPEAT AND RAPD ANALYSIS IN *ARTEMISIA ANNUA* L.

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ABSTRACT

In the present study, the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on callus cultures of *Artemisia annua* were evaluated in terms of its growth, chlorophyll, carotenoid, protein, phenol and flavonoid content and possible genetic variations using ISSR and RAPD markers. Callus cultures grown on MS medium supplemented with three different 2,4-D concentration (1.0, 2.0 and 3.0 mg 2,4-D L^{-1} MS medium) showed significant alterations in terms of callogenesis and growth. Callus grown on MS + 3.0 mgL^{-1} 2,4-D was found to impose highest degree of alterations and the callus width as well as the dry weight of 25 days old callus was highest at MS + 3.0 mgL^{-1} 2,4-D. Chlorophyll, carotenoid and flavonoid showed an increment in dose dependent manner while protein and phenol content declined as the concentration of 2,4-D increased in the medium. Investigations focused on DNA fingerprinting analysis using RAPD and ISSR markers showed genetic variations induced by high concentration of 2,4-D. Out of 31 markers used in this study, markers UBC-823, UBC-886, UBC-840, UBC-895 and OPE-7 showed polymorphism above 50%. These five markers showed their potential role in detection of genetic variability induced by high concentration of 2,4-D under *in-vitro* condition at an early stage and the maximum polymorphism was found in callus grown in highest concentration of 2,4-D i.e. MS + 3.0mgL^{-1} 2,4-D. Our results clearly demonstrated that in *A. annua* use of 2,4-D for callus generation above the concentration of 2.0 mgL^{-1} medium, may induce genetic variations and thus should be avoided where the need of generation of genetically alike callus/plantlets is desired.

KEYWORDS: 2,4-D, RAPD, ISSR, Callus, Metabolites, Genetic instability.

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INTRODUCTION

In recent years Plant cell/ Tissue culture techniques have emerged as a potential alternative to traditional agriculture for the mass production of secondary metabolites. Accumulation of secondary products in plant cell culture depends on the constituents and concentration of the culture medium. In the present study, *Artemisia annua* was taken as experimental plant. It is a Chinese medicinal herb known as Qinghao (green herb) and is the only natural source for the production of antimalarial compound artemisinin that plays a central role in combating drug resistant malaria, caused by *Plasmodium falciparum*. Since artemisinin was discovered as the active component of *A. annua* in early 1970s, most of the papers have focused on the medicinal properties of artemisinin and its potential role in malaria and cancer, Beside artemisinin, also herbal tea of *A. annua* are rich in antioxidant phenolics (mostly flavonoids)[1,2]. Few studies have reported the combination of a group of flavonoids such as quercetin, apigenin, luteolin, and kaempferol had both individual and synergistic effects against *Plasmodium falciparum in-vitro*[3].

A. annua is a plant with annual habit and the low concentration of its active component artemisinin in plant is the major limitation to satisfy its global demand. Maximum artemisinin concentration is present at pre-flowering or full bloom stage [4, 5] and extraction of this potential drug from plant takes around 7 – 9 months on an average [6]. In contrast to the drug extraction from the mature plants, plant cell culture technology for continuous and constant year round supply of natural active plant products; have been developed in some species of *Artemisia* such as *A. vulgaris*[7], *A. douglasina*[8], *A. Pallens*[9] and *A. annua*[10, 11]. Differentiation through callus cultures involves changes in some of the biochemical pathway[12]. In addition analysis of various cellular metabolites and enzyme activities in *A. annua* provide a reasonable and promising approach towards an understanding of the biochemical basis of the developmental and metabolic pathway[13]. Usually, auxins such as IAA (Indol-3-acetic acid), IBA (Indol 3-butyric acid), NAA (Naphthalene acetic acid) and 2-4,D (2,4-Dichlorophenxyacetic acid) are used for the induction of calluses and improvement of rooting. Out of these four synthetic auxins, 2,4-D has been extensively used as a growth regulators in plant cell culture and there are reports that high concentration of 2,4-D cause epigenetic and genetic variations in few plants [14] under *in-vitro* condition.

Optimizing reliable assays to assess the genetic variation at early stages of development are highly desirable. Various methods have already been used to characterise variation or mutation-derived cultivars such as ISSRs and RAPD[15, 16]. In the ISSRs techniques the detected polymorphism is based on the number of di-, tri- or tetra-nucleotide repeats whereas in the case of RAPD random primers are used. In both the cases no prior information on the sequence is required, with the help of RAPD and ISSRs reproducible polymorphisms are produced in high number. ISSRs primers have been used for assessing genetic diversity in various crop such as *Brassica juncea*[17], wheat [18], *Chrysanthemum* and *Citrus* pooled samples [19,20] and individual plants of oilseed rape cultivars[21] and flax anther culture-derived plants of microspore origin [22]. The present study was focused on the effect of 2,4-D under *in-vitro* condition in terms of growth, biochemical parameters and variation induced by high concentration of 2,4-D if any, in the callus cultures of *A. annua.*

MATERIALS AND METHODS

Preparation and establishment of callus culture

In the present study shoot apical meristem (SAM) and nodal segments were used as explants for *in-vitro* experiment. The explants were obtained from mature plant of *A.annua* maintained at the Botanical Garden, Department of Botany, Banaras Hindu University, Varanasi, India. These explants were sterilized with 70 % (v/v) ethanol and 0.1% (w/v) mercuric chloride. Surface sterilized explants were inoculated on full strength MS medium[23] aseptically. The medium was supplemented with 2, 4-dichlorophenoxy acetic acid (2,4-D) in different

concentrations i.e. 1.0, 2.0 and 3.0 mgL^{-1} of MS medium . The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were inoculated aseptically then incubated in culture room at 25 ºC and 16-h light followed by 8-h of dark period. Observations were taken at regular interval.

Biochemical Analysis

Callus obtained at different combinations of 2,4-D was used for various biochemical analyses. Accurately weighed powders of callus sample were ground with a pestle and mortar in the measured volume of solvents. The extract were filtered and separated through Whatman (No.1) filter paper. Each extract was prepared freshly for the analysis to prevent any degradation.

Estimation of Chlorophyll and Carotenoid

The Chlorophyll and carotenoid was extracted in 80% Acetone[24]. The absorption of the extracts at wavelengths of 663and 645 nm for chl and 480 and 510nm for carotenoid were measured with a SP 722E spectrophotometer. The concentrations of Chl *a* and Chl *b* were then calculated using the equations as follow.

Chl *a* = $12.7\ A_{663} - 2.59A_{645}$

Chl *b* = $22.9A_{645} - 4.67A_{663}$

Estimation of total Protein

The estimation of protein was done by Lowry method[25], 0.5 g of sample was crushed in 5 ml of 0.1M tris buffer, centrifuged and supernatant was taken and 10% TCA was added and again centrifuged and finally the pellet was dried and was dissolve in 0.1N NaOH. This solution was used for protein estimation. A standard calibration plot was generated at 650 nm using known concentrations of BSA. The concentrations of Protein in the test samples were calculated from the calibration plot and expressed as mg protein equivalent /g of sample.

Total Phenolic content

The total phenolic content of callus extracts of *A.annua* was determined according to the method described by Imeh and Khokhar[26]. Aliquots of the extracts were taken in a 10 ml glass tube and made up to a volume of 1.58 ml with distilled water. Then 0.1 ml folin-ciocalteau reagent and 0.3 ml Na_2CO_3 (20%) were added sequentially in each tube. A blue colour was developed in each tube because the phenols undergo a complex Redox reaction with phosphomolibdic acid in folin-ciocalteau reagent in alkaline medium which resulted in a blue colour complex, molybdenum blue. The test solutions were warmed for 30 min at 40 ºC, cooled and absorbance was measured at 650 nm. A standard calibration plot was generated at 650 nm using known concentrations of catechol. The phenol concentration was measured as mg catechol equivalents from a calibration curve with six concentrations of catechol.

Estimation of total flavonoids

The Aluminium chloride ($AlCl_3$) method was used for the determination of the total flavonoid content of the sample extracts[27]. Dry tissue was powdered and extracted with 25 ml of 95% of ethanol. Then 2 ml of extract was mixed with 0.1ml $AlCl_3$ (10%), 0.1 ml Potassium acetate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. Absorbance at 415 nm was recorded after 30 min of incubation. A standard calibration plot was generated at 415 nm using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample.

Genomic DNA extraction

DNA was extracted from each callus using CTAB method with minor modification[28]. The fine powder were transferred to a 50 ml Oakridge tube containing 15 ml pre-warmed extraction buffer (2% CTAB, 1.5 M NaCl; 20 mM EDTA, 100 mMTris, pH 8.0; 0.3% ß-mercaptoethanol added prior to use). After that, 5 µl RNAase (60 µg/ml) was added to it and this mixture was incubated at 65 ºC for 1 hour. Thereafter, equal volume of phenol: chloroform : isoamyl alcohol (25:24:1) was added to it and after mixing thoroughly for 15 min on shaker, it was centrifuged at 15000 rpm for 15 min at 20 ºC. After centrifugation, supernatants were transferred to a fresh Oakridge tube and chloroform : Isoamyl alcohol (24:1) was added. Samples were mixed thoroughly on shaker for15 min and centrifuged at 15,000 rpm for 15 min at 4ºC.

The DNA present in upper aqueous phase was precipitated in 2/3 volume of chilled isopropanol. After keeping the mixture at -20ºC for 30 min, it was centrifuged at 15,000 rpm for 15 min at 4 ºC. DNA was washed with 70% alcohol and dissolved in TE buffer (pH 8.0). The DNA content was determined by spectrophotometer (Gene Space-III, Hitachi, Japan) with optimal A_{260}/A_{280} ratio ranging from 1.80 to 1.95 and DNA quality was checked on 0.8% agarose gel.

Amplification conditions of ISSR and RAPD markers

ISSR analysis was performed in a final volume of 25 ul containing 25 mg DNA, 200 µm dNTPs, 20 pm of primer, 1unit of taq polymerase (FermentasInc, MD, USA), and 1 PCR reaction buffer (16mM (NH_2) SO_4, 67 mM Tris HCL) with 15 mM of $MgCl_2$. In total 23 ISSRs and 8 RAPD primers (Integrated DNA Technologies, IA USA) were tested. PCR amplification was programmed on Bio-Rad thermal cycler with 40 cycles of denaturation at 94 ºC for 1 min, annealing 55 ºC for 1 min, extension at 72 ºC for 2 min and final extension at 72 ºC for 10 min. PCR products were separated on 2% agarose gels buffered with 1×TAE (40 mM TRIS acetate, 1 mM EDTA pH 8.0) and stained with Ethidium Bromide. Gel images were acquired and analysed with Gel Doc 1000 (Bio-Rad).Primers used in this study were tabulated in Table 3.

Statistical Analysis

Statistical data analyses for the above experiments, three replicates were used for each treatment and each experiment was repeated thrice. The results are expressed as a Mean ± Standard error (SE) of 6 independent experiments. Statistical analysis was done by using the formula:

$$SE = \pm \sqrt{\frac{(X^2)}{n(n-1)}}$$

SE = Standard error
X = deviation of mean
n = number of replicates
The correlation coefficients were calculated as suggested by Searle [29].

RESULTS

Establishment of Callus and Regeneration

The MS basal medium without growth regulators failed to respond for callus induction from internode and shoot apical meristem as explant. Addition of growth regulators to MS basal medium resulted in callus induction but the frequency varied with the type and concentration of growth regulators used (Fig 1). However, when supplemented with 2,4-D, 50-87% callusing was observed at different concentrations (1.0 to 3.0 mgL^{-1}) with good growth of callus. Emergence of shoot from callus began 20 days after inoculation. This process was not completely synchronous and shoots initiation continued for several days in the cultures. There was de-novo regeneration of shoots from callus and different explants. Callus growth was found to be better at higher 2,4-D concentration (3.0 mgL^{-1}) as compared to low concentration (1.0 mgL^{-1}).

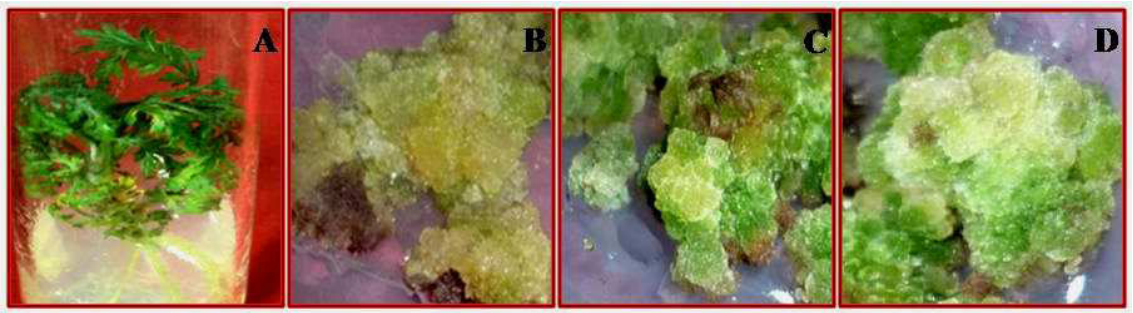

Figure 1

Callus regeneration from A. annua shoots (25 days after inoculation). (A) Shoot inoculated in Basal MS medium (B) callus regeneration in MS + 1.0 mgL^{-1} of 2,4-D (C) callus regeneration in MS+ 2.0 mgL^{-1} of 2,4-D and (D) callus regeneration in MS + 3.0 mgL^{-1} of 2,4-D.

Callus growth analysis

Shoot emergence response was observed to be high in basal MS medium in which no growth regulator was used i.e. 90% (Table 1). On the other hand, addition of 1, 2 and 3 mgL^{-1} 2,4-D to the MS medium resulted in 70 %,(75%) 50 % and 25 % shoot response respectively. Callus growth was vigorous when MS medium was supplemented with 3 mgL^{-1} 2,4-D and was about 4.5 cm (Table 2) in width. The estimates of correlation coefficient (Table 2) portrayed that the correlation were found positive as well as negative for all the traits thereby establishing strong inherent relationship among the attributes studied. The association of average number of branching were significantly positive with shoot emergence response, whereas significantly negative with callus width. Negative and significant relationship between callus width and shoot emergence response might be assigned to correlation factor.

Table 1
The impact of different growth regulators on shoot, callus and average branching of A. Annua

Growth Regulators	Response	Shoot emergence Response (%)	Callus width (cm)	Average Number of branches
2,4-D (1 mgL^{-1})	++++	75	3.5	20
2,4-D (2 mgL^{-1})	**+++**	50	4.0	15
2,4-D (3 mgL^{-1})	**++**	25	4.5	5
BASAL MS	+++++	90	1	25

Table 2
Correlation between growth regulators and shoot, callus and average branching in A. Annua

Characters	Shoot emergence Response	Callus width	Average Number of branches
2, 4-D (1 mgL^{-1})	1.00		
2, 4-D (2 mgL^{-1})	-1.00**	1.00	
2, 4-D (3 mgL^{-1})	0.50*	-0.53*	1.00

Significant at 5% probability level, **Significant at 1% probability level

Biochemical Profiling in terms of Pigments, Protein, Phenolics and flavonoids

Chlorophyll and Carotenoid content

Both chl *a* and *b* was found to increase with increasing concentration of 2,4-D. The amount of chlorophyll was higher in callus which was fully green and attained full growth. The amount of chlorophyll *a* was 1.61, 1.96, 2.64 mg/g FW respectively, whereas chl *b* was 0.548, 0.816 and 0.916 mg/g FW respectively in the callus regenerated on 1, 2 and 3 mgL^{-1} 2,4-D (Fig. 2A and 2B). The obtained data showed the strong correlation between Chl *a* and Chl *b* contents with increasing fresh weight. In tune with the chlorophyll response, Carotenoid content was also found to increase with increasing concentration of 2,4-D. The amount of carotenoid was 1.05, 1.56 and 1.57 mg/g FW respectively in the callus grown on MS media supplemented with 1, 2 and 3 mgL^{-1} 2, 4-D respectively (Fig. 2C).

Protein and Phenol content

Protein also show similar trend as phenol, total amount of protein present in callus regenerated on 1.0, 2.0 and 3.0 mgL^{-1} 2,4-D was 97.3, 72.9 and 23.6 mg/g FW respectively (Fig. 2D and 2E). The amounts of protein decreased as the concentration of growth regulator were enhanced. This was probably due the cells had stop differentiating or may not be metabolically active to utilize growth regulator. It was observed that total protein were higher during differentiation of callus rather in controlled callus. The total amount of phenol present in callus regenerated on medium containing 2,4-D i.e. in 1, 2 and 3 mgL^{-1} 2,4-D was 4.22, 4.07 and 3.70 mg /g FW respectively (Fig. 2 E). The phenol content decreases at the subsequent increase in the concentration of 2, 4-D. Thus it was observed that the callus which are differentiating contain minimum phenol as compared to callus which has stopped differentiating or in fully grown callus. Phenolicoxidations of the scared areas

of direct regenerated explants are important features on rooting success.

Total flavonoid content

Production of flavonoid in callus culture was found to be dependent on 2,4-D concentration. A steady rise in flavonoid content was observed from 1 mgL^{-1} to 3 mgL^{-1} 2,4-D. High flavonoid content was found in fully grown callus in which no further differentiation is possible i.e. from differentiating callus to undifferentiating callus. This may be due to high biomass accumulation which resulted in high flavonoid content. The amount of flavonoid was 0.040, 0.042, 0.044 mg /100 g DW on 1, 2 and 3 mgL^{-1} 2,4-D (Fig. 2F) respectively.

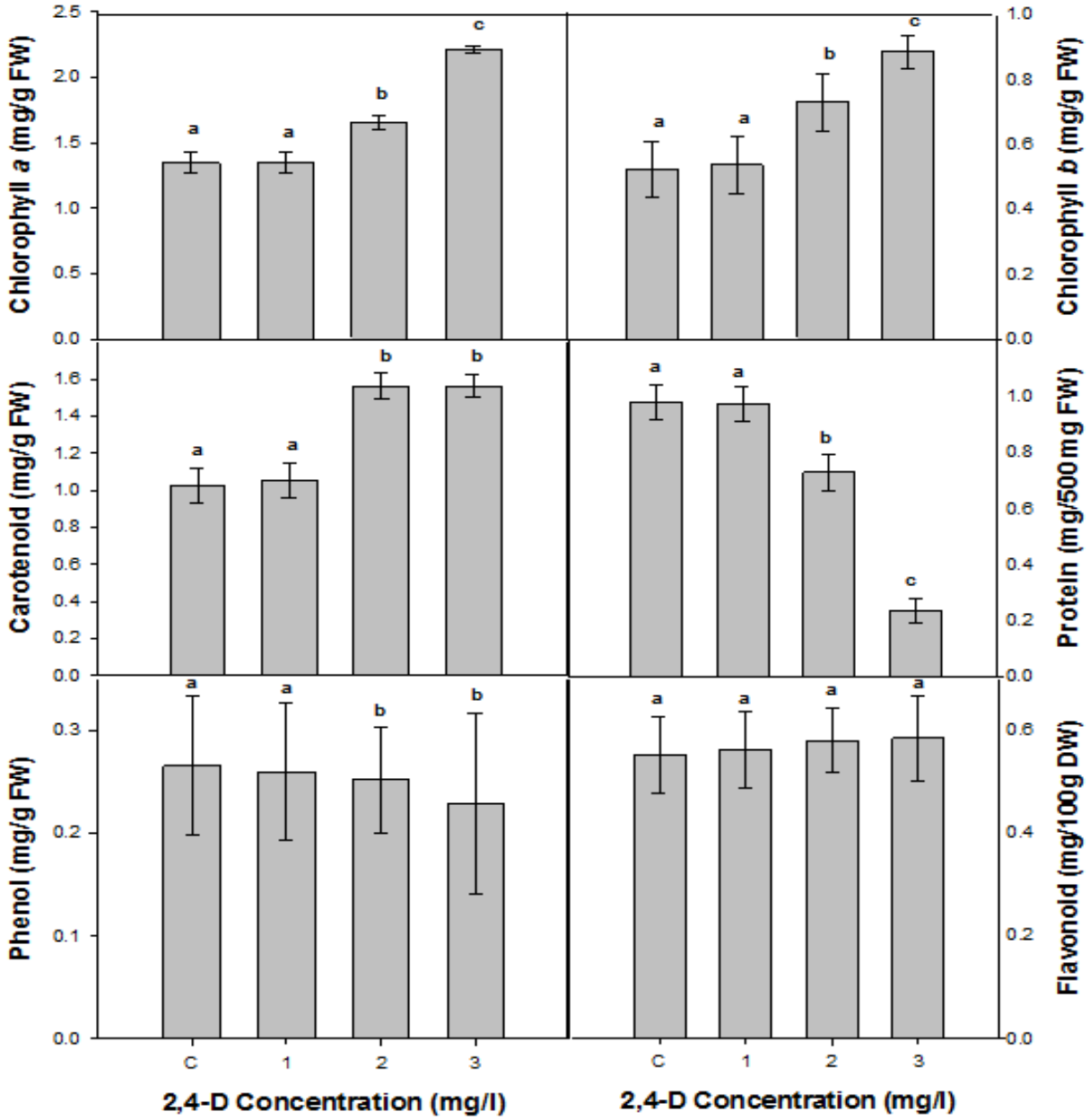

Figure 2

(A) Chlorophyll a (B) Chlorophyll b (C) Carotenoid (D) Protein (E) Phenol and (F) Flavonoids levels in callus of A. annua cultured on different concentration of 2,4-D. Each value represents the mean ± standard error (n=3). Means with different letters above bars were significantly different at the 0.05 level according to Duncan's multiple range test.

Molecular characterization using ISSR and RAPD Markers

Molecular characterization of DNA isolated from *A. annua* calli, grown on MS media supplemented with different 2,4-D concentration were performed using 23 ISSRs and 8 RAPD primers of variable length (15 or 16 nucleotides, and a G+C content within 25% and 66.6 %). All of them amplified scorable fragments ranging from 250 to 3000 bp, depending on the primer used. Out of them, markers UBC-823, UBC-886, UBC-840, UBC-895 and OPE-7 showed polymorphism above 50%. Obvious variations between *A. annua* calluses were observed. Genomic instability was evaluated for each primer using the Polymorphism Index Content (PIC). PIC values give an account of the bands generated from normal calli and of those altered in variant callus DNA. The data given in (Table 3)suggest an initiation of genomic instability at an early stage of calli formation. Consequently, such alterations occurred at higher rate during cellular proliferation this will resulting in a greater number of genomic lesions in calli at a later stage.

Table 3

DNA marker information, Resolving power and polymorphic information content (PIC) of 23ISSRs and 8 RAPD markers

Markers	Sequences	Total band	Polymorphic Band	Polymorphic %	Resolving power	PIC
UBC 810	GAGAGAGAGAGAGAGAT	3	1	33.33	0.5	0.15
UBC 814	CTCTCTCTCTCTCTCTA	1	1	100.00	0.5	0.94
UBC 815	CTCTCTCTCTCTCTCTG	3	1	33.33	1.5	0.60
UBC 823	TCTCTCTCTCTCTCTCC	2	1	50.00	1	0.38
UBC 822	TCTCTCTCTCTCTCTCA	3	1	33.33	0.5	0.15
UBC 824	TCTCTCTCTCTCTCTCG	2	0	0.00	0	0.00
UBC 834	AGAGAGAGAGAGAGACYT	2	0	0.00	0	0.00
UBC 836	AGAGAGAGAGAGAGACYA	5	2	40.00	3	0.45
UBC 825	ACACACACACACACACT	6	2	33.33	1	0.13
UBC 886	CTCCTCCTCCTCCTCCTC	6	3	50.00	0.5	0.07
UBC 889	DBDACACACACACACAC	5	3	60.00	3	0.48
UBC 841	GAGAGAGAGAGAGAGACTC	4	2	50.00	1	0.19
UBC 848	CACACACACACACACAAGG	9	3	33.33	2	0.19
UBC 809	AGAGAGAGAGAGAGAGG	3	1	33.33	1	0.29
UBC 840	GAGAGAGAGAGAGAGAYT	9	5	55.56	4	0.35
UBC 854	TCTCTCTCTCTCTCTCAGG	1	0	0.00	2	0.63
UBC 855	ACACACACACACACACYT	6	2	33.33	0.5	0.16
UBC 890	VHVGTGTGTGTGTGTGT	4	1	25.00	1	0.23
UBC 891	VHVGTGTGTGTGTGTGT	4	1	25.00	0.5	0.23
UBC 895	VHVGTGTGTGTGTGTGT	5	4	80.00	2.5	0.84
UBC 876	VHVGTGTGTGTGTGTGT	7	2	28.57	1.5	0.17
UBC 887	DVDTCTCTCTCTCTCTC	5	2	40.00	2.5	0.44
UBC 811	GAGAGAGAGAGAGAGAC	7	2	28.57	1.5	0.17
OPC 15	GACGGATCAG	10	3	30.00	3.5	0.56
OPD 7	TTGGCACGGG	7	3	42.85	2.5	0.47
OPD 8	GTGTGCCCCA	6	2	33.33	1.5	0.29
OPD 11	AGCGCCATTG	6	1	16.66	1.5	0.20
OPD 13	GGGGTGACGA	6	1	16.66	0.5	0.11
OPD 20	ACCCGGTCAC	5	2	40.00	1.5	0.41
OPE 1	CGCAAGGTCC	6	2	33.33	2.0	0.51
OPE 7	AGATGCAGCC	4	2	50.00	1.0	0.39

Fingerprint patterns were obtained for one control 'Normal' calli and three 2,4-D treated calli. The normal calli was obtained on basal MS medium whereas these treated calli had been obtained on MS medium supplemented with different concentrations of 2, 4-D, *viz.* 1, 2 and 3 mgL^{-1} 2,4-D. Variations in fingerprinting patterns obtained by primer UBC-810 resulted in the occurrence of total three bands of 500, 750, and 1000-bp for the control, first treated calli (1 mgL^{-1}) and second treated calli (2 mgL^{-1}) (Fig. 3) but on the other hand the third treated calli (3 mgL^{-1}) was characterized by the loss of 250-bp marker. The primer UBC-836 resulted in total of four bands of 400, 500, 650 and 900-bp in control and first treated calli whereas in second and third treated calli there was loss of 650-bp and 900-bp markers respectively. The primer UBC-889 resulted in total four bands of 500, 700, 750 and 1500-bp in control, first and second treated calli sample but third treated sample was characterized by loss of 1500-bp marker. The primer UBC-886 resulted in total of five bands of 500, 650, 1000, 1500 and 2000-bp in control and first treated calli whereas in second treated calli there was loss of 1500 and 2000-bp markers and in third treated calli there was also loss of 1500 and 2000-bp markers but an additional 750-bp marker was observed which was absent in control and other two calli. Similarly out of 8 RAPD primers used in this study, 3 primers (OPD-7, OPD-20 and OPE-7) showed polymorphism above 40% (Fig.4). Primer UBC-810, UBC-836, UBC-889, UBC-886 and OPE-7 enabled the detection of polymorphisms in one control and three treated calli and confirmed the genetic variation of the same callus. The polymorphism between calli was clear, as shown in Table 3. The extent of variations observed in second and third treated calli samples were higher than that obtained within control and first treated calli sample.

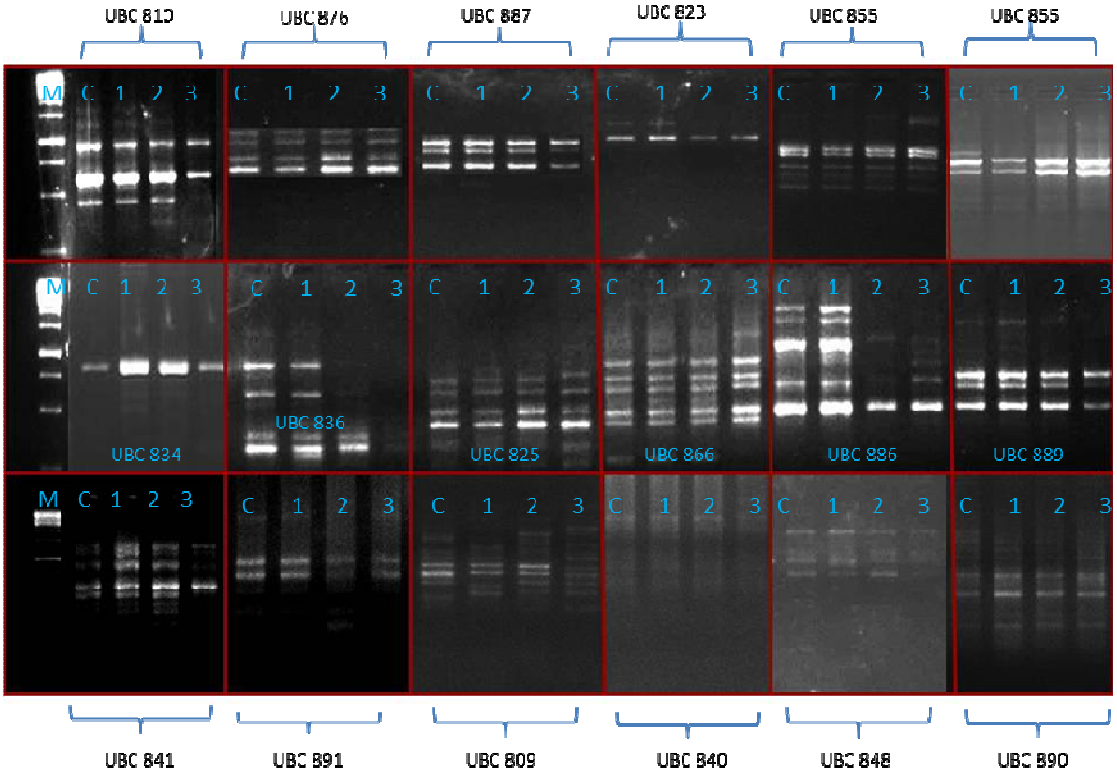

Figure 3

Banding profiles of four A. annua calli generated from the same mother plant i.e. one control (C) and three variant calli (1, 2 and 3 mg/L 2,4-D treated). Amplification products from the ISSRs reaction were obtained using primers indicated by arrow. First and last lanes indicate 1 Kb marker.

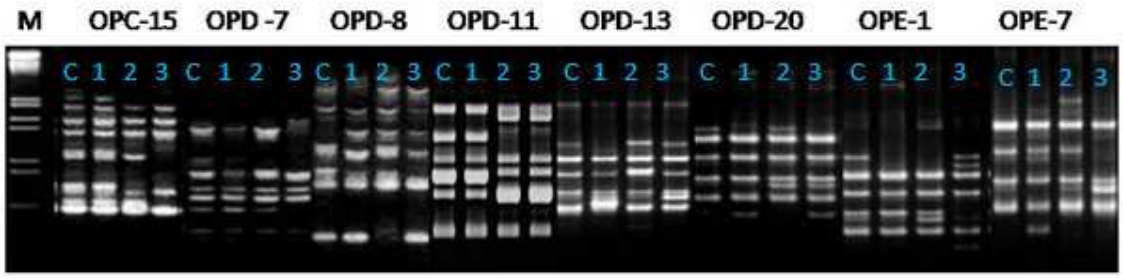

Figure 4

Banding profiles of four A. annua calli generated from the same mother plant i.e. one control (C) and three variant calli (1, 2 and 3) generated by 8 RAPD primers. First and last lanes indicate 1 Kb marker.

DISCUSSION

In the present study it was observed that the frequency of callus induction increased with the increasing concentration of 2,4-D in the medium. This is due to the fast rate of cell division induced by 2,4-D. Presence of 2, 4-D has been shown to be essential for callus formation in many plants for example *Momordica charantia*[30]. The important factor is oxidizing or metabolizing reaction for the success of callus organogenesis. The role of auxins in callus induction was also advocated in *Stevia rebaudiana*[31] in *Prosopis cineraria*[32] and in *Celastrus paniculatus*[33]. Both chlorophyll and carotenoid increased significantly in a dose dependent manner in 2,4-D supplemented medium. In plants, they contribute to the photosynthetic machinery and protect them against photo-damage as chl *b* is associated with photosystem II, the high chlorophyll content would possibly increase the efficiency of photosystem II which play an important role in photosynthesis. The concentration of chlorophyll and carotenoid increase significantly in the callus culture supplemented with 2,4-D in the medium and was highest in calli cultured on MS + 3 mgL^{-1} 2,4-D. Changes in the chlorophyll content due to 2,4-D could be related to induction of callus growth rates and its primary and secondary metabolic activities [34]. Phenols are one of the main secondary metabolites produced by the plant and have been reported to have multiple biological effects, including antioxidant activity [35]. Continuous decrease in phenolic content in the callus was observed with the subsequent increase in the concentration of 2,4-D in the medium and was minimum in callus cultured on 3 mgL^{-1} 2,4-D medium followed by callus cultured on 2 mgL^{-1} 2,4-D medium. As the callus starts differentiating at concentration 1 mgL^{-1} and/or 2 mgL^{-1} 2,4-D in the medium, the cells are highly active, dividing and quantitatively changing their activities and when the concentration of 2,4-D reached its maximum i.e. 3 mgL^{-1} no more cell differentiation took place and growth stopped. The phenol concentration is high before differentiation. As reported by Mader and Fusel[36] the decrease in phenolic content during differentiation is probably due to its involvement in cross-linking of cell wall constituents which is catalysed by peroxidase.

Flavonoids are the most common group of polyphenol compounds that are found commonly in plants. These are widely distributed in plant and are involved in many functions. They possess anti-hepatotoxic, anti-allergic, anti-inflammatory, anti-osteoporotic, and antitumor, anti-proliferative, and anticancer activities [37, 38, 39]. In this study we found a significant increase in the concentration of flavonoids with the increase in the concentration of 2,4-D in the medium and was maximum in callus cultured on 3 mgL^{-1} 2,4-D medium. Addition of 2,4-D and kinetin into the media was also found to enhance the flavonoids production in *Genista tinctoria*[40]. The presence of growth regulator in combination with 2,4-D was beneficial for valeportiate accumulation in *Valeriana gelechomifolia* callus culture[41]. Similar results have been recorded for other secondary

metabolites [42]. In general low concentration of 2,4-D in the medium stimulate DNA, RNA and Protein synthesis in plants whereas at higher concentration it induces RIP (repeat induced point mutation) ultimately causing dramatic elevation of cytosine methylation in plants[43]. An increase in 2,4-D concentration in carrot suspension cultures from 0.5 to 2 mgL^{-1} raises the percent 5-methylcytosine from 16% to 40%[44]. However, 2 mgL^{-1} is commonly used concentration of 2,4-D in monocot tissue cultures. The growth regulators may affect tissue culture variations by causing a general increase in methylation[14]. However, growth regulator such as 2,4-D may act differently. The increased transcription can also alter the chromatin structure. These alterations could disrupt the stability/expression of the genome[14]. Various molecular mechanisms are responsible for the DNA mutation and genetic instability leading to the development of variations [43]. In the present study therefore ISSR and RAPD markers have been used to detect the variants at the DNA level to assess genomic variability. In the present investigation, ISSR primers UBC-810, UBC-836, UBC-889 and UBC-886 and among the RAPD primer OPE-7 (Fig.3) was found to be more informative in detecting genomic instability/variations in *A. annua* calli at an early stage. In addition, ISSR technology detected significant levels of DNA polymorphism at the first callus phase; this result proves that this approach constitutes a fast and efficient technique in the assessment of variations obtained through tissue culture. The alteration observed in the fingerprinting pattern due to the loss of a part of a gene involved in the regulation of cell proliferation may be a consequence of microsatellite instability at the primer binding site, or due to modification(s) at poly –GT SSR or between the microsatellites. However, all these results demonstrate that the use of microsatellites is more complex. Our results are in contrast to the results obtained by Wolff et al [18] where they used RAPDs and inter-SSR PCR for detecting polymorphism in DNA from *in-vitro* or vegetatively propagated plants. They found no RAPD or SSR-PCR differences between phenotypically different members of a family, which led them to suppose that they had failed in showing polymorphism.The results obtained in our study not only suggest that genetic variations may take place during the callogenesis but also illustrate how the impact of growth regulator on DNA fingerprint patterns can be accessed through the use of microsatellite and RAPD primers. Such variations may, indeed, result from changes in either the growth regulator composition of the media used or an undifferentiated cell step. Another proposed explanation is that the plants regenerated from unorganised callus vary more than those from organised callus, whereas no or very little variation occurs when plants are regenerated directly without an intermediate callus phase [45]. However, in tune with our results, Fisher et al [46] also observed the presence of additional internal microsatellites for some of the ISSR markers. Such an observation would imply a clustering of microsatellites in some genomic regions. From DNA fingerprinting of in-*vitro* grown callus, it has been suggested that strand slippage during DNA replication may be the major cause of the length polymorphism by the addition or deletion of a few repeats at a time in the genome.

CONCLUSION

The data reported here highlight that genomic variation can be detected at a very early stage under *in-vitro* conditions in *A. annua*. Four of the twenty three microsatellite primers tested proved to be efficient in revealing clear changes in banding profiles. ISSRmarkers require less DNA, generate a high number of markers and are highly reproducible, which make them a candidate of choice in the study of plant genome stability/variability. Changes in the levels of metabolites during differentiation from callus culture can be useful in our understanding of biochemical basis of developmental pathway during callogenesis in *A. annua.*

ABBREVIATION

2,4-D: 2, 4-Dichlorophenoxyacetic acid
ISSR: Inter simple sequence repeats
SAM: Shoot apical meristem
RAPD: Randomly amplified polymorphic DNA
Chl *a*: Chlorophyll *a*
Chl *b*: Chlorophyll *b*

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