Project Title- "Role of Polyphenols during somatic embryogenesis and expression of polyphenol oxidase gene in *Plantago ovata* Forsk, during development"

F.No.41-508/2012 (SR) Dated- 17-07-2012

Report of the work done-

Brief objective of the project-

- Establishment of suspension culture of *P.ovata* to induce somatic embryogenesis(SE).
- Estimation of total polyphenol content by FCR method from differentiating callus(DC) and non - differentiating callus(NDC).
- Determination of contents of polyphenols eg. Gallic acid, Ferulic acid ,Rutin, Caffeicacid,cinnamic acid ,quercetin , Trans- Resveratrol, Vanillic acid, Synergic acid etc by HPLC analysis during somatic embryogenesis from DC and NDC by spectrophotometric analysis.
- Cloning and sequencing of PPO gene expressed during somatic embryogenesis (SE).
- Bioinformatics study of the PPO gene.

Summary-

Plantago ovata is a good source of polyphenols and polyphenols have great health benefits .So experiments were set up to increase its amount through tissue culture.Four combinations of kinetin and 2,4-D (Group A, Group B and Group C and Group D) [(0.5 mg/L Kinetin and 1 mg/L 2,4D) , (1 mg/L Kinetin and 0.5mg/L 2,4D), (1mg/liter kinetin and 1mg/liter 2,4D) and (0.5mg/L Kinetin and 0.5 mg/L 2,4D) respectively were used in MS media for callus induction. Quantity of total polyphenols and Total antioxidant are relatively higher in 21 days callus of combination Band C and comparatively low incombination A and D. the study depicted that,higher Kinetin concentration had a positive impact in polyphenol accumulations and total antioxidant. Polyphenol oxidase (PPO) enzyme causes tissue browning by oxidation of phenolic group, so its overexpression was not acceptablein tissue culture. Hormonecombination of group B proved beneficial in respect of rise in polyphenol accumulation and down-regulation of PPO expression. The beneficial role of Kinetin in enhancing polyphenol accumulation was further verified with another experimental set up where Kinetin and 2,4-D as PGRs was applied in combination like [Group I (control) (0.5 mg/L Kinetin and 0.5 mg/L 2,4-D) , Group II (1 mg/L Kinetin and 0.5mg/L 2,4-D), Group III(1.5mg/L kinetin and 0.5mg/liter 2,4-D) and Group IV (2.0 mg/L Kinetin and 0.5 mg/L 2,4-D) in MS media for callus induction. Kinetin was applied in ascending concentration gradient from 0.5mg/L to 2mg/L and 2,4-D of fixed concentration i.e. 0.5mg/L. Increment in kinetin concentration induced more polyphenol accumulation, total flavonoid and total antioxidant activity in 21 days old *Plantago ovata* callus. Gradual increase in total polyphenols and total flavonoid from Group I to III and decline in Group-IV occurred. Total antioxidant activity increased gradually from Group I to IV. Beneficial Polyphenols like (+)-Catechin, vanillic acid, Rutin, Luteolin 7-O-β-D-glucoside and Trans-cinnamic acid were detected and quantified by HPLC technique. First step of the phenylpropanoid pathway is catalyzed by PAL enzyme which leads to the formation of phenolic compounds.RT-PCR and qPCR technique were used to find PAL gene expression which got up-regulated significantly in group II and III, significant down- regulation of PPO gene expression occurred with increment of kinetin application to that of the control during callogenesis, which was the advantageous output of the experiment. The study continues with sub-culturing 21 days callus with (1.5mg/L and 2mg/L) and fixed 2,4-D (0.5mg/L) for non- embryogenic callus induction and on the other hand with NAA and BAP(0.5 mg/L and 5mg/L) respectively for embryogenic callus induction. Histology study of the embrogenic callus showed globular somatic embryo formation of P.ovata. A comparative analysis was carried out to find Polyphenol content and total antioxidant activity

of 48 days old non embryogenic callus, embrogenic callus and 63 days embrogeniccallus.PAL and PPO expression was analyzed accordingly. Elicitors induce Polyphenols accumulation. Polyphenols also get increased in presence of stress. Plants experience stress due to heavy metal pollution caused by improper discarding of the industrial waste. Hexavalent chromium is one of the heavy metal pollutants in India and also present particularly to some region of India, where Plantago grows to a great extent. The aim of the study was to find the effect of hexavalent chromium on *P.ovata* and how the plant responded to such heavy metal exposure *in vitro*. Morphological changes in the reduction of shoot and root length were significant in a dose-dependent manner from low to high. However multiple root development took place at 100 μM, 300 μM and 500 μM doses. *P.ovata* (10 days) showed tolerance response up to 1500μm concentration in respect of rising in secondary metabolites accumulation(polyphenols), Chlorophyll content(Chlorophyll a, b, total chlorophyll), carotenoids and total anti-oxidant activity but DPPH radical scavenging activity were not significantly high with Cr(VI) doses. MDA (Malondialdehyde) content was significantly low, depicting low lipid peroxidation, and showed tolerance against chromium stress. Stress-induced gene (*PPO* and *PAL*) expression showed significant upregulation as compared to the control. *PAL* gene expression showed upregulation till 1500 μM dose and *PPO* gene significantly up to 1800 μM, highest with 1000 μM concentration. Atomic absorption spectroscopy technique depicted chromium accumulation in the shoot (0-1800 μM) and root (0-500μM). Chromium accumulation in shoot and root of *P.ovata* (ppm) increased in a significant manner with increasing potassium dichromate concentration in the germination medium. Though the morphological changes in terms of reduction in shoot and root length occurred in a dose-dependent manner from low to high, but to some extent, *P.ovata* combated the stress significantly by inducing the stress responses and showed tolerance against chromium stress.

- Structural prediction was carried out of the partial sequence of the PPO.

Introduction-

Plantago ovata Forsk commonly known as Isabgul is an herbaceous plant. It has its origin in Europe and then to southern Mediterranean to Eastern Asia including India, Iraq, Spain and Canary is-lands. The production of *Plantago ovata* seeds is commercialized and widely produced in European countries, Pakistan and India.[Ross, 2005].The seed husk of Pysillium is widely used as a remedy for dysentery and intestinal disorder.(Dhar et al. 2005) This plant also possess many other medicinal uses and its uses are from ancient times .Many countries like India, Iran, Spain and Thailand has their traditional way of using as medicinal plants.(Ross,2005). It is used to improve kidney and bladder function, as diuretic, etc. (Zargari, 1994). It has many therapeutic values and helps in the treatment of constipation, irritable bowel syndrome, diarrhea, inflammatory bowel disease, colitis, Hypercholesterolemia, colon cancer and diabetes. (Singh, 2007)

Apart from all the medicinal uses, *Plantago ovata* is a good source of plant secondary metabolites such as Polyphenols. (Talukder et al. 2015). Polyphenols are a class of heterogeneous bioactive compounds produced in many medicinal and vegetable plants during secondary metabolism. Polyphenols are mainly characterized by the presence of phenolic groups in the molecules. The position of hydroxyl or phenol group in the polyphenolic compounds are believed to determine their antioxidant properties, especially against peroxyl and superoxide radicals (Rice-Evans et al., 1996). Significant amount of Polyphenols accumulation are also seen in callus culture of P.ovata. (Talukder et al. 2015).Because of the wide use of medicinal plants, researchers are interested to explore it in many ways. In vitro tissue culture is a popular techniques to enhance the amount of plant secondary metabolities (Hirose et al. 1990; Lee Y et al. 2011).Plant growth regulators are used to induce callogenesis during in vitro callus culture. The effectiveness of the growth of the callus and accumulation of polyphenols as plant secondary metabolities depends on the growth regulators and its concentration (Deus and Zenk 1982; Lee et al.2011). Somatic embryo induction is the artificial way of embryo formation and in the process of tissue culture polyphenol accumulation is the natural phenomenon. Some studies revealed its role in maintaining efficiency of the embryo and some polyphenols specially get enhanced in some species indicating as a marker for SE induction in that species (Kouakou et al 2007). Phenylalanine ammonia-lyase (PAL,) is the enzyme which catalyzes the first step in the

Phenylpropanoid pathway and it's over expression leads to increase in accumulation of Polyphenols. (Chang et al. 2009). Increase in the content of the plant secondary metabolities help plant to withstand biotic and abiotic stress. Biotic and abiotic stresses lead to the enhancement in accumulation of plant secondary metabolites. (Baslam et al. 2013). Enzymes of the phenylpropanoid pathway take part in the synthesis of several polyphenols and with the synthesis of polyphenolic compound there occur an increase in susceptibility of the phenolic group to get oxidized by Polyphenol oxidase, (PPO,EC 1.10.3.2),a gene whose gene product is responsible for the formation of brown coloration in plant tissue. It's an enzyme containing copper and causes hydroxylation of monophenolsto form O-diphenols. O-diphenols oxidized to O-quinones, forming the brown pigmentation (Mazzafera and Robinson 2000; Dirks- Hofmeister et al. 2014). Many higher plants possess PPO as an active enzyme, but it has no definite biological function, many predicted possible functions were proposed by many authors.(Mazzafera and Robinson 2000; Mayer 2006; Li et al. 2017). Phenolics are susceptible to oxidation by Polyphenol oxidase and leads to the formation of brown pigment. So when several elicitors and plant hormones are being used to enhance the accumulation of Polyphenols by tissue culture technique, simultaneous expression of PPO gene causing oxidation reaction is also an area of interest (Mayer and Herel 1979; Ferrar and Walker, 1996; Walker and Ferrar, 1998).

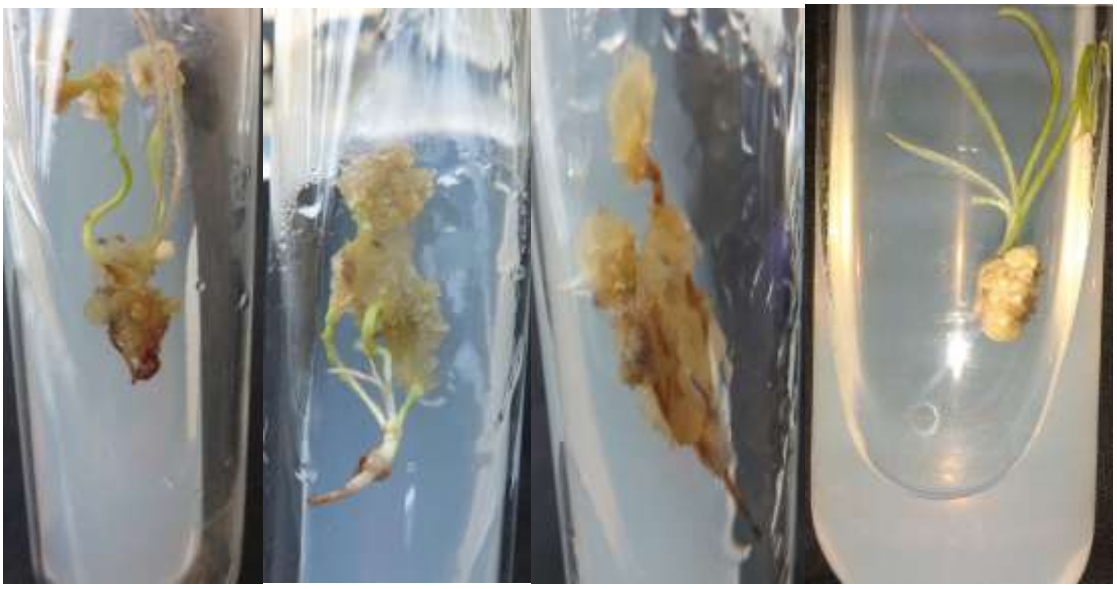
Chapter 1- Establishment of callus culture with different combinations of hormone and its effect on polyphenol accumulation and *PPO* gene expression.

Introduction-

Callusing is induced by plant growth regulators. Cytokinin(Kinetin) and Auxin (2,4D) have their significant role in callus induction. In this study four combinations of hormones are used to enhance polyphenol accumulation in 21 days P.*ovata* callus culture. In this paper we found some correlation between Polyphenol accumulation and external kinetin exposure during callus culture of *Plantago ovata.* Polyphenol oxidase gene expression is not always correlated to total polyphenol content.[10] .Ascorbic acid content is significantly high in Loquat food treated with kinetin.[15].So effect of Kinetin can be correlated with increase in Total antioxidant.

RESULTS

Figure 1-21 days P.*ovata* callus with four combinations of Kinetin and 2,4D in MS media.



GroupA (0.5mg/L Kinetin)+(0.5mg/L 2,4D)	Group B(1mg/L Kinetin)+(0.5mg/L 2,4D)	Group C(1mg/L Kinetin)+(1mg/L 2,4D)	Group D (0.5 mg/L Kinetin + 1mg/L 2,4-D)

Figure 1- shows the 21 days callus of *Plantago ovata* Forsk. Browning of callus is comparatively high in Group C and D.

Figure 2- **Total Polyphenol Content**

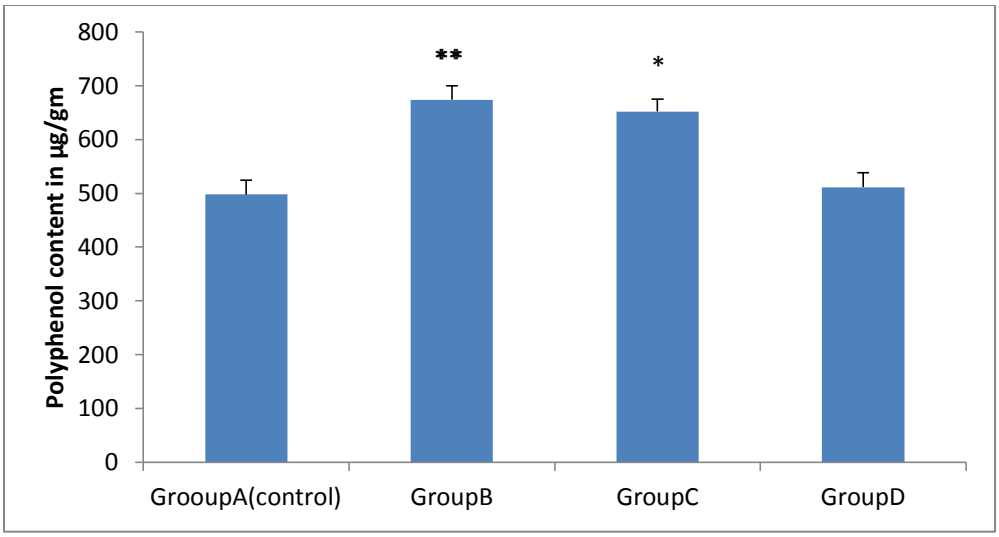


Figure 2-Group A-(0.5mg/L Kinetin)+(1mg/L 2,4D),Group B-(1mg/L Kinetin)+(0.5mg/L 2,4D),Group C-(1mg/L Kinetin)+(1mg/L 2,4D),Group D-(0.5mg/L Kinetin)+(0.5mg/L 2,4D)

Total Polyphenol content is relatively high in group B and group C but relatively low in Group A and Group D. Result depicts that high Kinetin level in the media has some positive impact in accumulation of Polyphenols.

Figure 3- **Total Antioxidant**

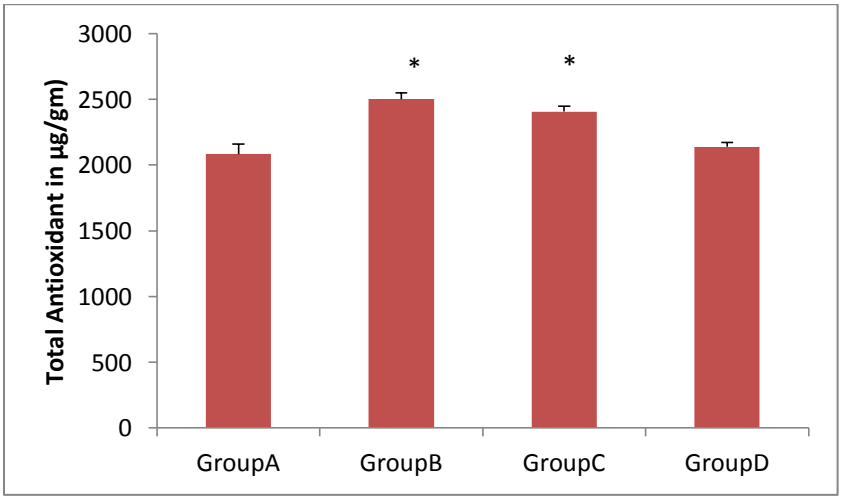
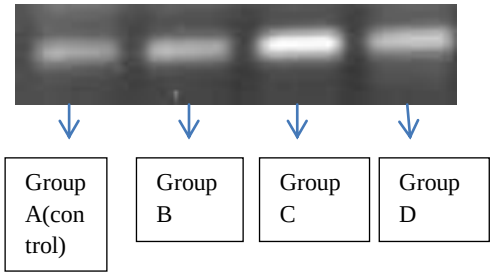
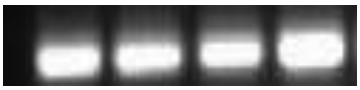


Figure 3 -Group A-(0.5mg/L Kinetin)+(1mg/L 2,4D),Group B-(1mg/L Kinetin)+(0.5mg/L 2,4D),Group C-(1mg/L Kinetin)+(1mg/L 2,4D),Group D-(0.5mg/L Kinetin)+(0.5mg/L 2,4D)

Total anti-oxidant is relatively higher in Group B and C. MS Media supplemented with higher kinetin concentration that is 1mg/L has some positive effect and has a direct correlation with Total antioxidant .

Figure4- Relative PPO expression





β Actin as endogenous control

Figure 5- Band Intensity

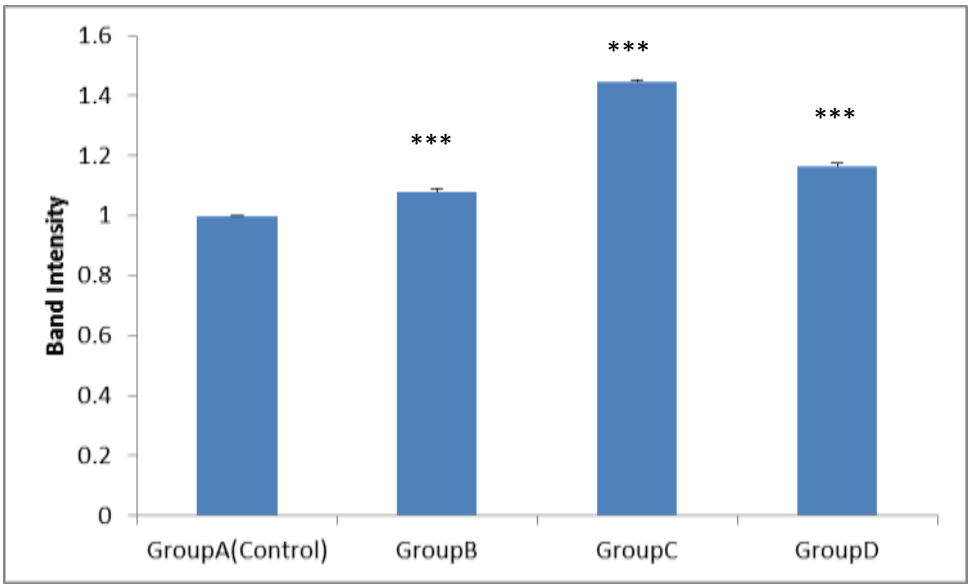


Figure5: Band Intensity -Group A-(0.5mg/L Kinetin)+(1mg/L 2,4D),Group B-(1mg/L Kinetin)+(0.5mg/L 2,4D),GroupC- (1mg/L Kinetin)+(1mg/L 2,4D),Group D-(0.5mg/L Kinetin)+(0.5mg/L 2,4D)

PPO gene expression is relative to the callus browning observed in 21 days old callus given in Figure-1. This result is relevant to many studies on enzymatic browning caused by Polyphenol oxidase enzyme. [10].Several papers have reported that high polyphenol accumulation is susceptible to PPO action.[2]. Polyphenol content and PPO action is not always correlated. Studies on several apple cultivars have shown that some cultivar with high Polyphenol content are not relatively high in PPO enzyme action. PPO enzyme action was found relatively higher in other cultivar with comparatively less Polyphenol content.[14] Densitometry of the PPO expression suggests Group B combination of the hormone shows negative correlation with Polyphenol content and Total antioxidant, which can be related to several studies [14].

Conclusion-

Polyphenol has diverse medicinal value. Its accumulation as plant secondary metabolities is a well-known fact. Plant biologists have shown diverse interest in isolating Polyphenols and its application in medicinal science. Tissue culture proves to be an important process of producing

polyphenols. *Plantagoovata* is known to provide several health benefits. Its tissue culture results in accumulation of Polyphenols in significant amount. Kinetin, as plant growth regulator has positive role in inducing polyphenol accumulation. Its application is beneficial to increase Polyphenol accumulation in significant amount. Polyphenol oxidase is known as Browning enzyme. Its activity results in tissue browning, which proves problematic economically in food industries. Polyphenol oxidase enzyme action causes callus browning during tissue culture. Kinetin, in several studies proves to improve tissue health by increasing ascorbic acid content and total polyphenol content. This study shows the Positive role of kinetin in plant tissue culture.

Chapter 2- Kinetin induced Polyphenol Accumulation and expression of Phenylalanine Ammonia-Lyase gene (*PAL*) and Polyphenol oxidase gene (*PPO*) in *Plantago ovata* Forsk in vitro.

Introduction-

The effectiveness of the growth of the callus and accumulation of polyphenols as plant secondary metabolities depends on the growth regulators and its concentration (Deus and Zenk 1982; Lee et al.2011). Kinetin and 2,4-D are two effective plant growth regulators which are used widely in *in vitro* callus culture. (Bevan and Northcote 1979).Kinetin, a cytokinin has many biological effects on cell cycle, altered expression of genes, in inhibiting auxin action and enhancing calcium flux. It has been reported that Kinetin has anti-stress, anti-aging properties and it self an antioxidant (Barciszewski et al. 2000).In this study, Kinetin is applied in ascending concentration gradient along with fixed 2,4-D concentration in four groups to induce callogenesis. There are evidences which document the fact that there are two plant growth promoting hormones i.e. auxin and cytokinin which are required to induce callogenesis and it's a widely accepted and popular use in plant tissue culture and horticultural field.(Ikeuchi et al. 2013).In our study the combination (0.5mg/L Kinetin and 0.5mg/L2,4-D) mimicked as the control group as other combinations such as (0.1mg/L Kinetin and 0.1mg/L 2,4-D),(0.3mg/L kinetin and 0.3mg/L 2,4-D) and (0 mg/L Kinetin and 0.5mg/L 2,4-D) did not result in fairish growth of callus which can be grant as the control group and to use further for all the experimental work. So Group I with (0.5 mg/L Kinetin and 0.5 mg/L 2,4-D) proved to be the best among these four combinations as control group and then keeping the 2,4-D concentration fixed, Kinetin concentration varied in

concentration in ascending order in Group II, III and IV, where increment of Kinetin concentration resulted in regulation of polyphenol accumulation, PAL gene and PPO gene expression in *P.ovata*. Another gene named Polyphenol oxidase (E.C. 1.10.3.2) found in nature, its gene product or the PPO enzyme cause the enzymatic browning in many species.(Li et al. 2017) The inactivation of PPO is required to minimize product losses caused by browning (Chen et al.2000; Le et al. 2004). In some reported studies, kinetin application causes reduction in tissue browning(Shyamali and Kazumi 2007; Alderson and Nagarajan 2012) and PPO gene activity(Vernon and Straus,1972)PPO also acts as an anti-oxidative defense enzyme in many plant species(Goud and Kachole 2012)

Objectives: Enhancement of Polyphenols by kinetin application is the prime objective of the study and simultaneously PPO gene product causes the oxidation of the phenolic groups leading to tissue browning, so study of PPO gene expression along with Polyphenol accumulation and upregulation of PAL with different Kinetin concentration are the lookouts of the experiment.

Results

The aim of this experiment is to initiate the callogenesis in three groups namely Group I-(0.5mg/L Kinetin +0.5mg/L 2,4-D), which was the control group ;Group II-(1mg/L Kinetin+0.5mg/L 2,4-D); Group III-(1.5mg/L + 0.5mg/L);Group IV-(2mg/L Kinetin + 0.5mg/L 2,4 D). *P.ovata* seedlings of 10 days were inoculated in the MS media to get the 21 days *P.ovata* callus to carry out the different experiments. Simple observing the calluses in four groups(Fig.1), we noticed *P. ovata* callus of Group I and II were comparatively more brown in coloration than Group III and IV, which were comparatively lighter.

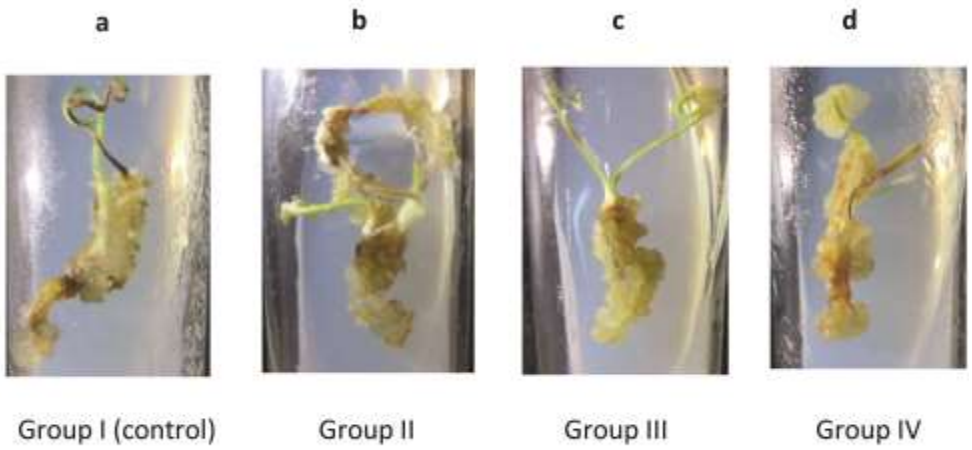


Fig.1 21 days *Plantago ovata* callus supplemented with different concentration of Kinetin and fixed concentration of 2,4-D. Group I- (0.5mg/L Kinetin +0.5mg/L 2,4-D) ;Group II-(1mg/L Kinetin+0.5mg/L 2,4-D); Group III-(1.5mg/L + 0.5mg/L);Group IV-(2mg/L Kinetin + 0.5mg/L 2,4 D)

Significant increase in callus mass observed in group IV (Fig. 2), where as in group II-III there was no such significant increase in fresh weight of the callus mass to that of the control group I.

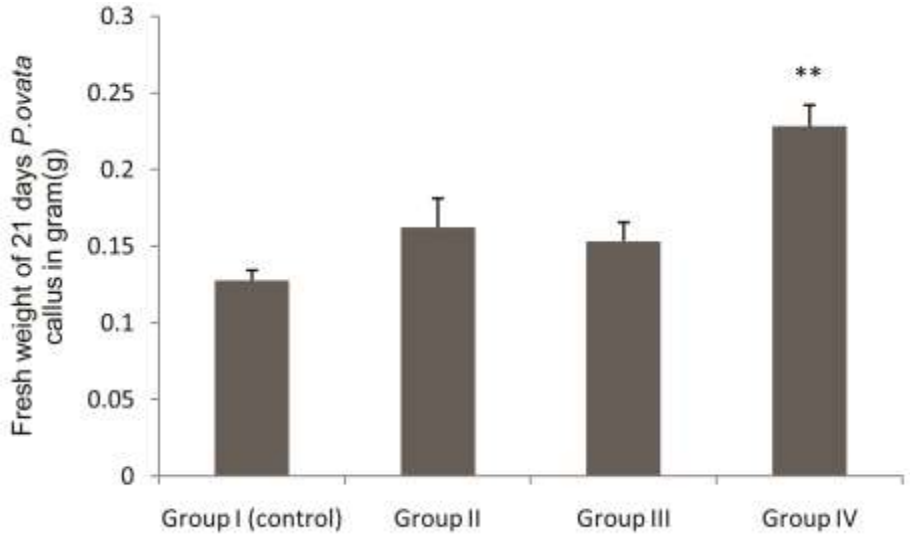


Fig. 2 Fresh weight of callus in gram (g).

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Total Polyphenol Content

In this study there was an objective to understand the effect of kinetin in accumulation of total polyphenols by tissue culture technique in *P.ovata*callus.Total Polyphenol content was determined in four groups of 21 days *P.ovata* callus by folin-ciocalteureagent(Fig.3). Total Polyphenol content increased significantly in Group-II and Group-III from the control. Highest accumulation of Polyphenols occurred in Group-III of about 764.77± 20.24 µg GAE g-1 FW which was 1.44 times more to that of the control. Though there was a significant increase in total polyphenol content in Group-II and Group-III from the control, no significant accumulation of total polyphenol occurred in Group-IV.

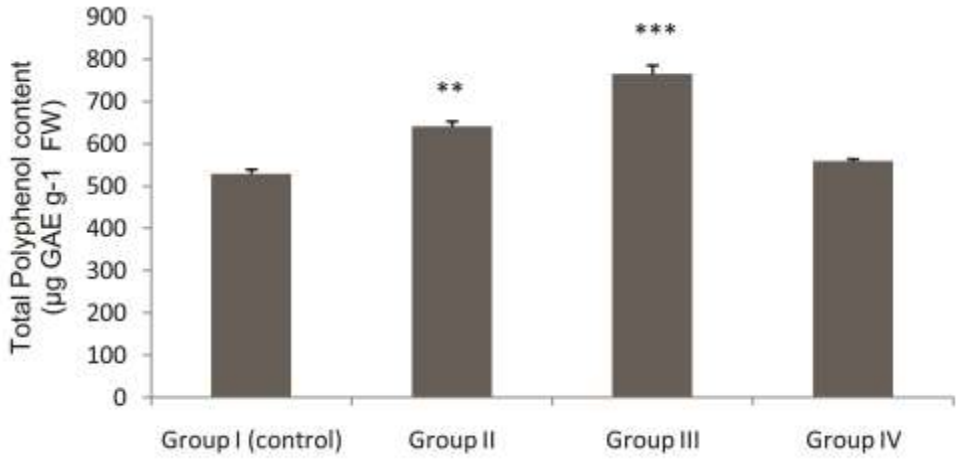

Fig.3 Graph representing difference in Total Polyphenol content in four groups of 21 days *P.ovata* callus exposed to different concentration of Kinetin and fixed concentration of 2,4-D during *in vitro* callus culture. **Group I**(0.5mg/L Kinetin +0.5mg/L 2,4-D) ;**Group II**(1mg/L Kinetin+0.5mg/L 2,4-D); **Group III** (1.5mg/L + 0.5mg/L);**Group IV**(2mg/L Kinetin + 0.5mg/L 2,4 D). Significance level is indicated with asterisks, *P <0.05; **P <0.01; ***P <0.001.

Total Flavonoid Content-

Kinetin was found to have a positive effect in accumulating polyphenols in the previous experiment and Flavonoid is a member of the Polyphenolic groups in the polyphenol classification and it has many health benefits as discussed earlier. So its accumulation was quantified byaluminium chloride (AlCl3) colorimetric method (Fig.4).Total flavonoid content increased significantly from the control like total Polyphenols in Group-II and Group-III, in Group-IV flavonoid content got declined in concentration in comparison to Group-II and Group-III from the control. Like Total Polyphenol content, highest accumulation of Total Flavonoid took place in Group-III supplemented with 1.5mg/L of Kinetin and 0.5mg/L 2,4-D. There was a 2.06 times increase in Total flavonoid content in Group-III from the control (Group-I) i.e. 203.836 ± 1.65 µg RE g-1 FW.

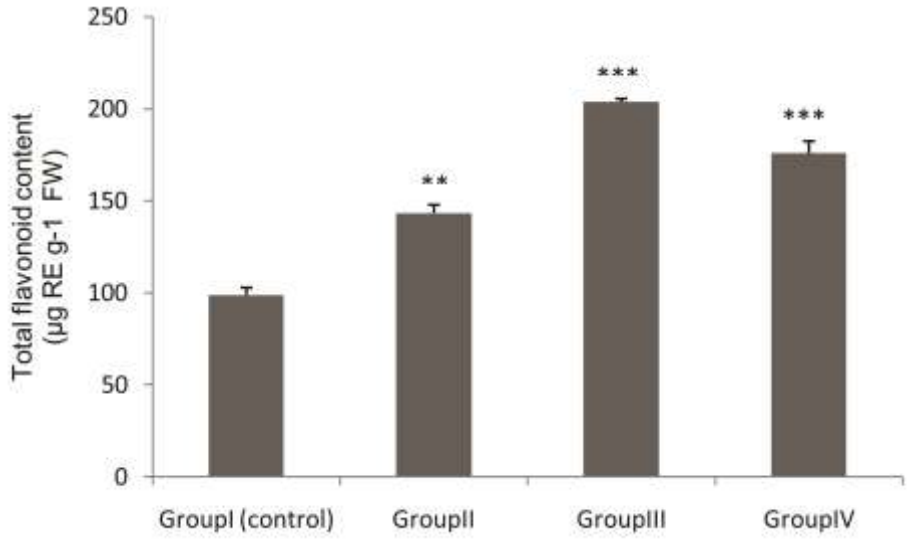

Fig.4 Total Flavonoid content in 21 days *P.ovata*callus during *in vitro* callus culture. **Group I**(0.5mg/L Kinetin +0.5mg/L 2,4-D) ;**Group II**(1mg/L Kinetin+0.5mg/L 2,4-D); **Group III**(1.5mg/L + 0.5mg/L);**Group IV**(2mg/L Kinetin + 0.5mg/L 2,4 D). Significance level is indicated with asterisks, *P <0.05; **P <0.01; ***P <0.001.

Total Antioxidant Activity

Folin–Ciocalteureagent (FCR) and aluminiumchloride (AlCl3) colorimetricmethod had inferred total polyphenol and flavonoid accumulation in the study. Now the level of increment of the anti-oxidant activity in the four groups is a concern. Phosphomolybdenum assay method had depicted the result (Fig.5) and there was a gradual significant increase in total antioxidant activity from the control from Group-II to Group-IV. Unlike Total Polyphenol content and Flavonoid content, highest antioxidant activity was found in Group-IV *P .ovata* callus. Total Antioxidant activity was 286.48± 7.60 µg AAE g -1 FW in Group-IV ,found to increase in 1.56 times from the control (Group-I).

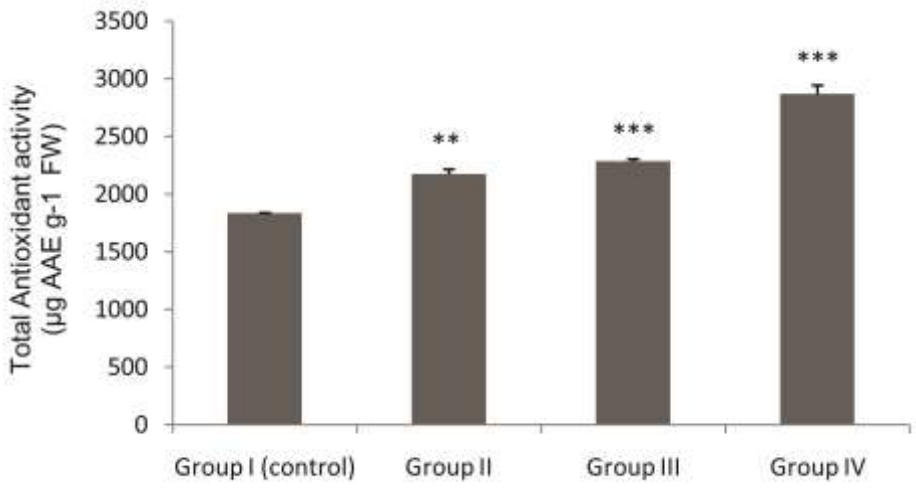

Fig.5 Total Antioxidant activity in 21 days *P.ovata*callus during *in vitro* callus culture.**Group I (**0.5mg/L Kinetin +0.5mg/L 2,4-D);**Group II**(1mg/L Kinetin+0.5mg/L 2,4-D);**Group III**(1.5mg/L + 0.5mg/L);**Group IV**(2mg/L Kinetin + 0.5mg/L 2,4 D). Significance level is indicated with asterisks, *P <0.05; **P <0.01; ***P <0.001.

Quantification of some beneficial polyphenols by HPLC analysis.

Polyphenols like (+) catechin, Vanillic acid, Luteolin 7-O- beta D glucoside and Trans-cinnamic acid were detected and quantified at wavelength maxima of 278nm, 260 nm, 324 nm,278 nm

respectively by HPLC technique. Use of Kinetin as PGR in tissue culture to enhance the accumulation of polyphenolsis the prime concern of the study. Each specific Polyphenols were detected comparing with the peak of the standard sample at specific wavelength and retention time (Rt). Polyphenols were measured calculating the peak area by comparing with the standard curves of the known polyphenols. These polyphenols were measured in µg/g FW of the callus tissue of the *P.ovata*. How kinetin is affecting in enhancement in accumulation of each of these polyphenols were quantified (Table 1). Specific polyphenol peak was detected at specific retention time in the HPLC chromatograph. (Fig.6).

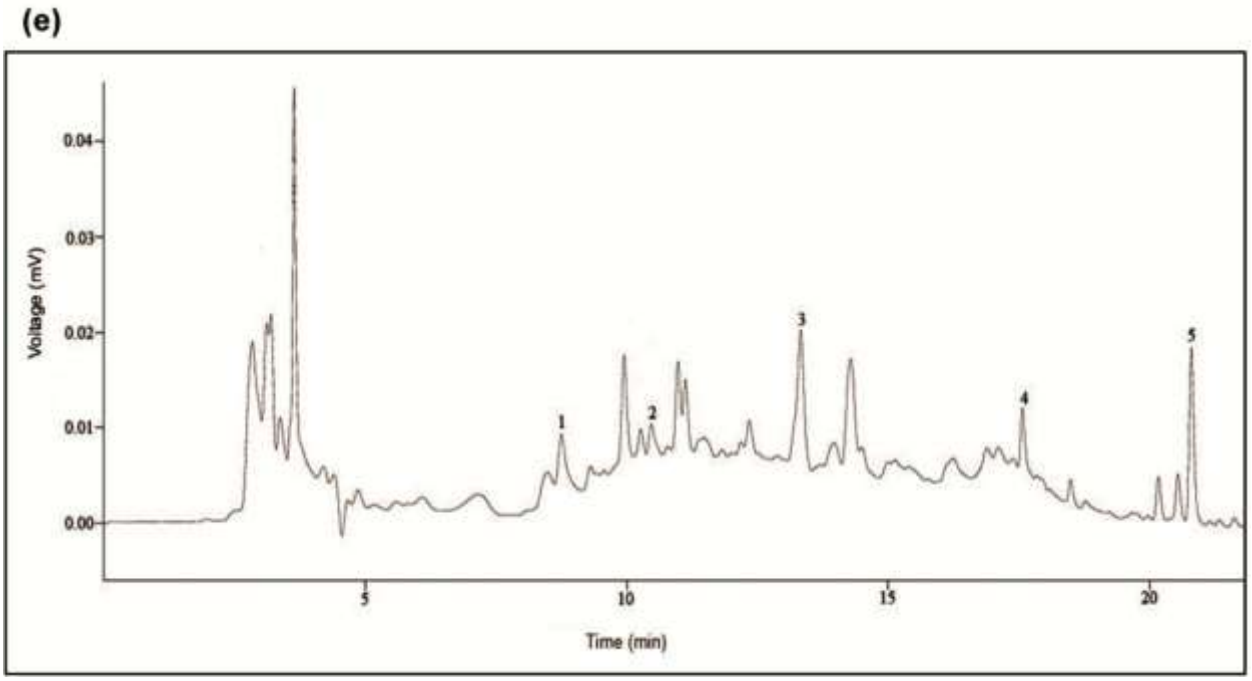

Fig.6 HPLC chromatogram showing polyphenol peak at specific retention time, peak **1** (+)-Catechin ; **2** Vanillic acid; **3** Rutin **4** Luteolin 7-O beta glucoside **5** Trans-cinnamic acid. HPLC chromatogram **a** group I (control) at 356nm **b** group II at 278 nm **c** group III at 278 nm **d** group III at 278 nm **e** group IV at 278 nm.

Table 1 Quantification of the beneficial polyphenols of the four groups of *P.ovata* callus tissue in µg/g FW of the callus tissue of the *P.ovata*

Beneficial Polyphenols	Concentration (μg/g) fresh weight of the callus tissue, mean ± S.D				Retention Time (min)	Wavelength maxima(nm)
	Group I	Group II	Group III	Group IV	8.3	278
(+)- Catechin	114.2±10.49	114.94±13.88	128.51±4.25	107.17±14.39		
Vanillic acid	6.01±0.84	8.5±0.63	10.24±0.22	13.50±1.6	10.8	260
Rutin	38.09±2.94	55.62±0.094	66.095±0.431	51.75±2.68	13	356
Luteolin 7-O-β-D-glucoside	22.35±0.775	31.533± 0.236	37.95±0.82	35.38±1.09	16.9	324
Trans-cinnamic acid	3.73±0.714	5.49±1.56	61.82±0.84	29.94±3.76	20.8	278

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Relative Expression of *PAL* gene-

Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenylpropanoid pathway and this pathway leads to the synthesis of polyphenolic compounds (Chang et al. 2009) so the regulation of expression of the PAL gene by different kinetin concentration in the medium during callogenesis was an objective of the study.PAL gene expression was studied by RT-PCR technique and the band intensity (Fig.7 a) was measured in image j software to get the densitometry(Fig. 7c). The result depicted the effect of kinetin in regulating the expression

pattern in four groups.

There occurred a significant increase in PAL gene expression in Group II and Group III i.e. 1.2 and 1.15 fold to that of the control respectively. Group IV showed decrease in relative expression as compare to Group II and Group-III.

The expression pattern of the PAL gene by RT-PCR technique was quantified by qPCR (Fig.7d). There occurred 1.8 and 1.08 fold increase in relative PAL gene expression from the control in group II and III. Relative PAL gene expression got decreased in Group IV P.ovata callus during in vitro callus culture

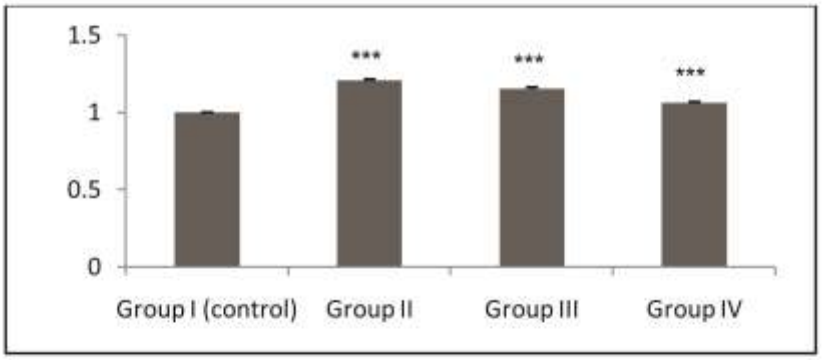
(a)

Group I Group II Group III Group IV

(b)

Group I Group II Group III Group IV

(c)



(d)

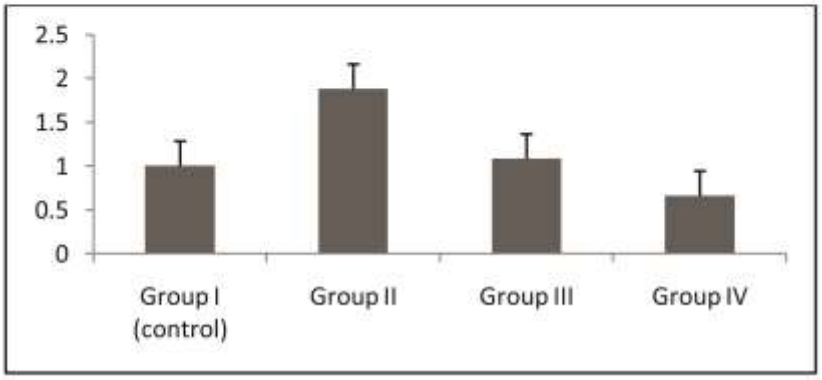


Fig.7 Relative expression of *PAL* gene **a** Gel picture of PAL gene expression in four groups **b** expression of endogenous control beta actin **c**Densitometry of Phenylalanine ammonia lyase (PAL) gene expression. Densitometry was done in image j software**d**Real-time quantitative PCR analysis of PAL gene expression in four groups with Kinetin in ascending concentration gradient from group-I to Group- IV. Data representation in fold change (mean±S.d) from the control (value1).Value represent as means of three replicates ± S.D. beta Actin used as endogenous control to normalize the data.

Relative Expression of *PPO* gene.-

The objectives of the study demanded the above experiments to be done to have the idea of the kinetin effect. Along with the expression analysis of the PAL gene, it was our concern to observe the Polyphenol oxidase (*PPO*) expression with the enhancement of the polyphenol accumulation by using kinetin. As discussed earlier PPO is responsible for tissue browning, and it oxidizes the phenolic group causing browning of the tissue. PPO gene expression was studied by RT-PCR technique and the band intensity (Fig. 8a) was measured in image j software to get the densitometry (fig: 6). the result unfold the effect of kinetin in regulating the expression pattern in four groups.

The expression pattern of the PPO gene was quantified by q-PCR (Fig. 8).Exogenous Kinetin application in ascending concentration gradient caused decrease in fold change of expression of PPO gene. There were 0.164, 0.467 and 0.387 fold change in Group-II, Group-III and Group-IV respectively from the control Group-I (value-1).

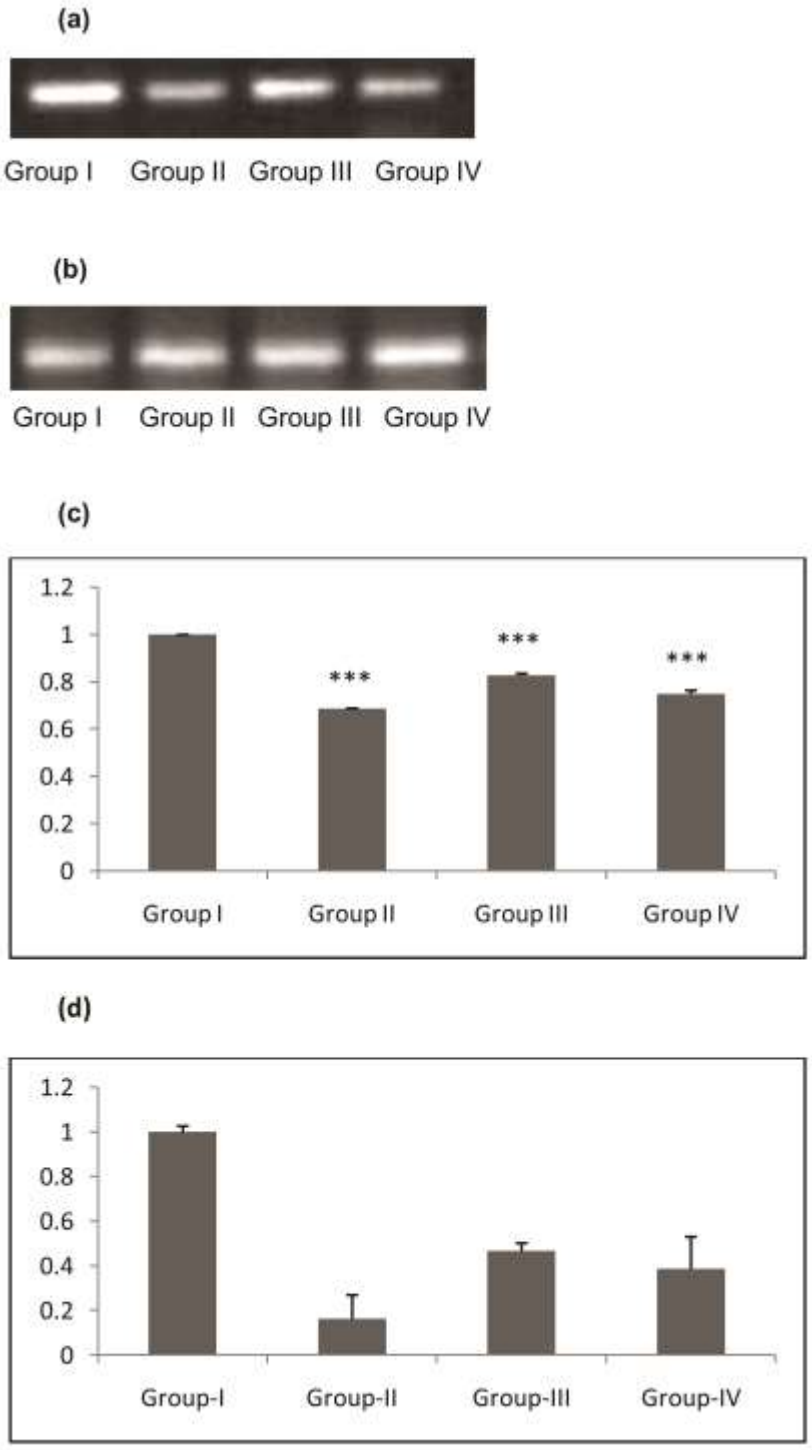


Fig.7 Relative expression of *PPO* gene **a** Gel picture of *PPO* gene expression in four groups **b** expression of endogenous control beta actin **c**Densitometry of Phenylalanine ammonia

lyase(*PPO*) gene expression. Densitometry was done in image j software **d** Real-time quantitative PCR analysis of *PPO*gene expression in four groups with Kinetin in ascending concentration gradient from group-I to Group- IV. Data representation in fold change (mean±S.d) from the control (value1).Value represent as means of three replicates ± S.D. beta Actin used as endogenous control to normalize the data.

Conclusion :

Kinetin proved to be an effective plant growth regulator in enhancing polyphenol accumulation by *in vitro* tissue culture technique.

In this study at optimum concentration, significant increase of polyphenol accumulation occurred in 21 days *P.ovata* callus. This technique can be utilized commercially to get beneficial polyphenols**.**

Chapter- 3: A comparative study on polyphenol accumulation in embryogenic and non-embryogenic callus.

Introduction-

Somatic embryogenesis induction is an artificial way of generating plant embryo from the vegetative part of the plant. This helps in plant propagation. Somatic embryo (SE) can be used as an artificial seeds for conserving endangered plant species.

Accumulation of phenolic compound is an usual phenomenon during callogenesis, it's also get accumulated in somatic embryo. Many studies corroborated that accumulation of phenolic compound has a role to play in increasing the efficiency of the somatic embryo (Wu et al. 2004)

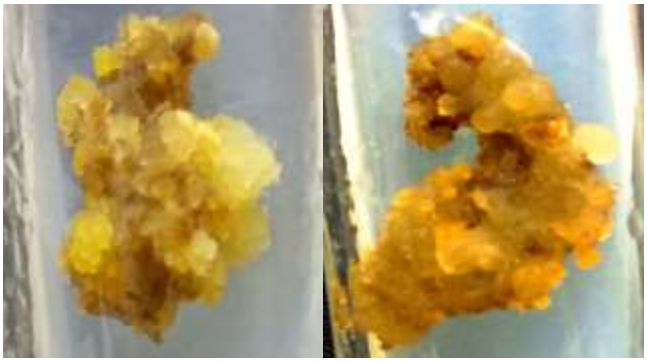
Objective of the study-

- To establish a comparative study in respect to polyphenol accumulation in both embryogenic and non embryogenic callus.
- To detect and quantify some beneficial polyphenols in both non-embryogenic and embryogenic callus**.**

Result-

Tissue culture for inducing 48 days *P.ovata* callus

21 days non-embryogenic callus were sub-cultured for another 21 days with (1.5mg/L kin +0.5 mg/L 2,4-D) and (2mg/L kin + 0.5 mg/L 2,4-D) respectively as P2, GIII and P2, GIV.



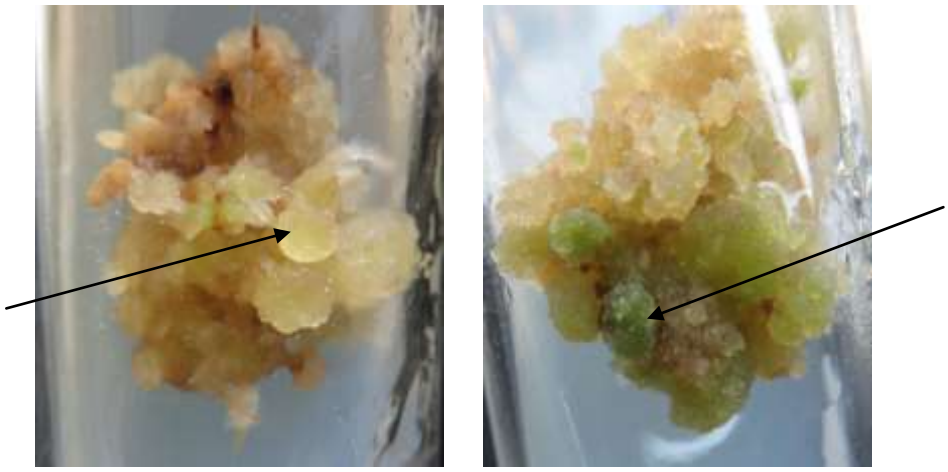
GIII GIV

Figure 1 : 48 days non-embryogenic *P.ovata* callus

Tissue culture for inducing somatic embryogenesis-

48 days Somatic embryogenesis were induced by sub-culturing 21 days non-embryogenic *P.ovata* callus on MS media with NAA and BAP at concentration (0.5 mg/L NAA and 5 mg/L BAP). 48 days somatic embryos were further sub-cultured with NAA and BAP on MS media to get 63 days old P.ovata somatic embryos.

Globular somatic embryos were formed, embryos got more solid in texture and green in color after sub-culturing 48 days old somatic embryo callus for 21 days on MS media with NAA and BAP at given concentration.



a b

Figure 2: a- 42 days somatic embryo of *Plantago ovata* , b- 63 days somatic embryo of *Plantago ovate*

Histological analysis of globular stage *P.ovata* somatic embryo.

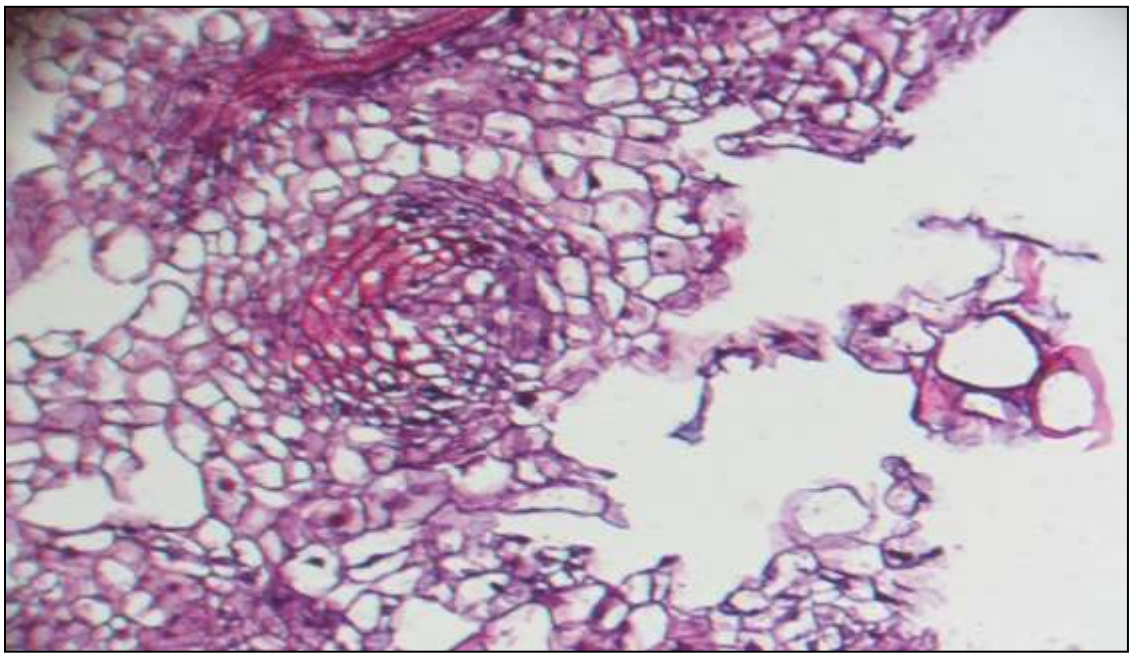


Figure 3 : Somatic embryo histological structure

Total Polyphenol estimation-

Total polyphenol content was higher in group III compare to group IV in 48 days non-embryogenic callus,1.5mg/L kinetin proved to be more effective than 2mg/L kinetin for polyphenol enhancement (Fig 4)

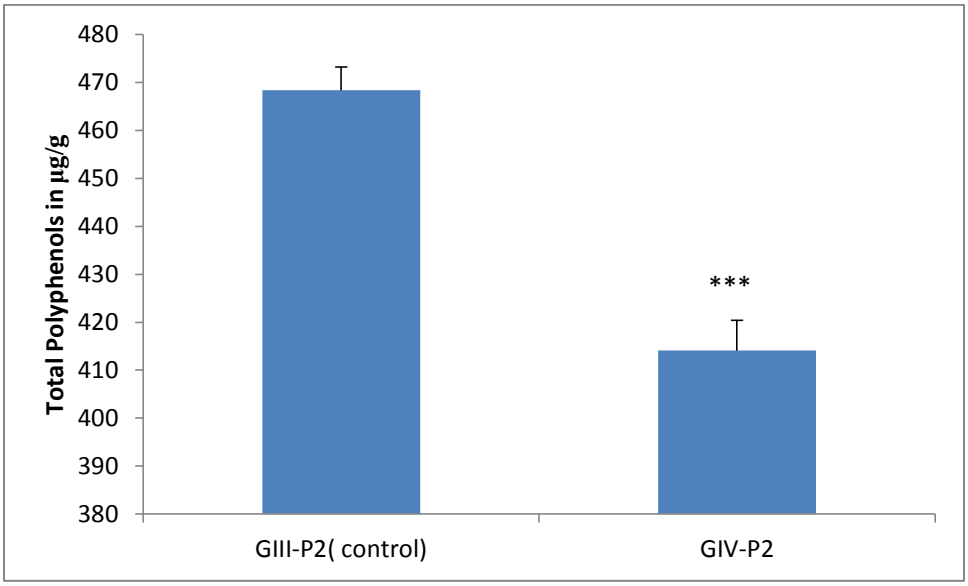


Figure 4: Total polyphenol content in 48 days old callus at hormone concentration of (1.5 mg/L kin +0.5mg/L 2,4- D) and (2mg/L Kin +0.5mg/L 2,4-D) named as GIII-P2 and GIV-P2 respectively.

As GIII-P2 found to be accumulating more polyphenols than GIV-P2, It was set as control group to analyze on comparative account of polyphenol accumulation between embryogenic and non-embryogenic callus.(Fig 5)

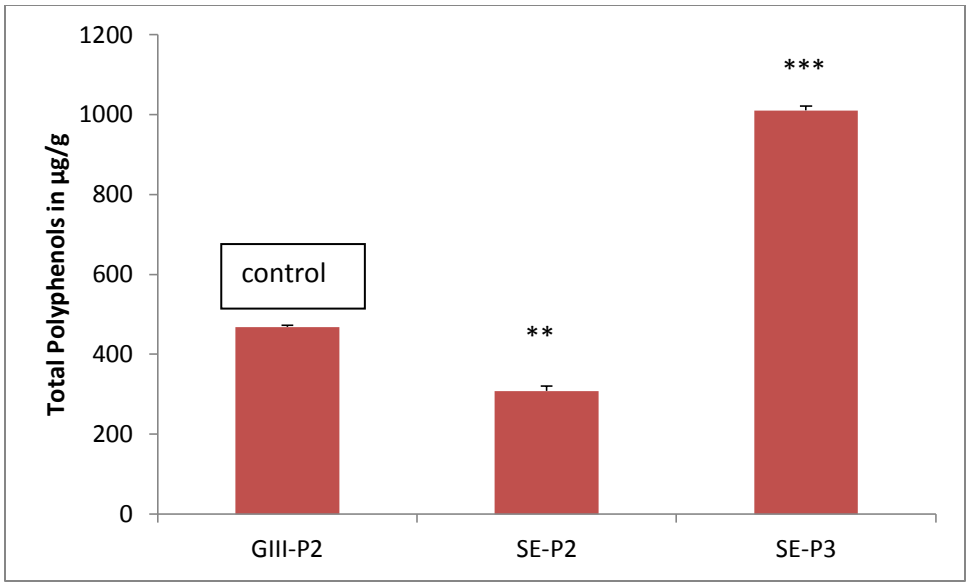


Figure 5- Comparative analysis of total polyphenol accumulation among control group named GIII-P2 (48 days non- embryogenic callus with 1.5 mg/L kin and 0.5 mg/L 2,4-D), SE-P2(48 days somatic embryo with NAA and BAP at concentration 0.5 mg/L and 5 mg/L respectively) and SE-P3(63 days somatic embryo with same hormone supplementation as SE-P2).

Total antioxidant activity-

Unlike total polyphenol accumulation total antioxidant is comparatively higher in GIV-P2 than GIII-P2. This is similar to the previous study, where antioxidant activity was higher in GIV in 21 days non-embryogenic callus. (Fig 6)

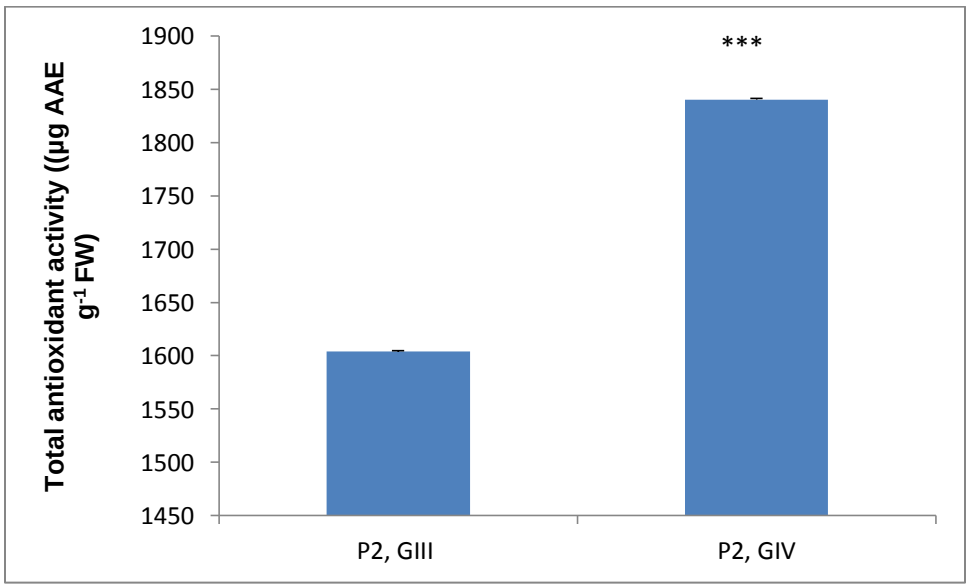


Figure 6: Total antioxidant in 48 days non-embryogenic callus.

Total antioxidant is comparatively low in 48 days somatic embryo, but increased in 63 days old somatic embryo(SE) callus. In Fig 7, GIII-P2 was taken as control and significant rise of total antioxidant was observed in 63 days old SE callus. Similarly when non-SE GIV callus was granted as the control group, result depicted significant increase in total antioxidant activity in 63 days SE callus (Fig 8).

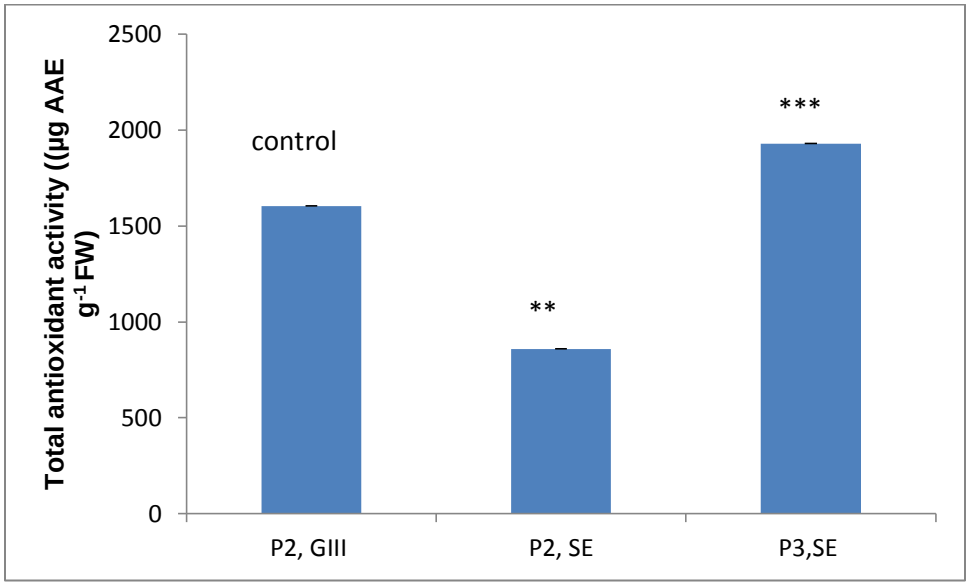


Figure 7: Total antioxidant activity in 48 days non-SE(control)(P2-GIII), in 48 days SE(P2-GIII) and 63 days SE(P3-SE).

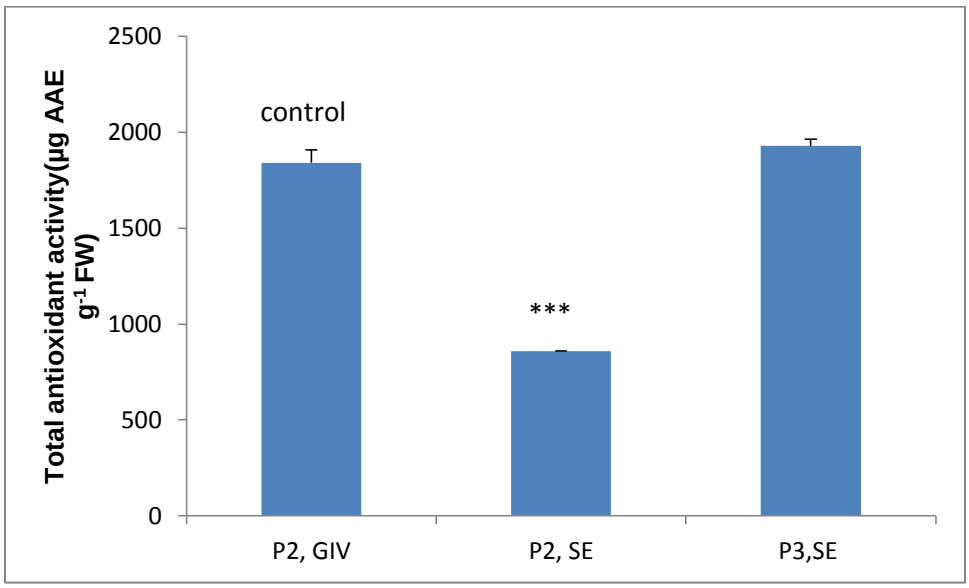


Figure 8: Total antioxidant activity in 48 days non-SE(P2-GIII), in 48 days SE(P2-GIII) and 63 days SE(P3-SE).

Expression analysis of PAL and PPO gene

Up-regulation of PAL and PPO expression occurred in SE callus compare to non-SE callus.

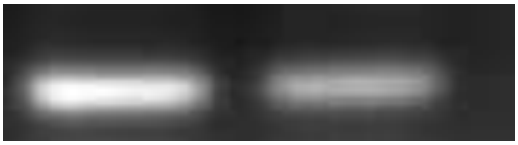


Figure 9: PAL *gene* expression in GIII and GIV 42 days old callus.(lane 1- GIII and lane 2- GIV)

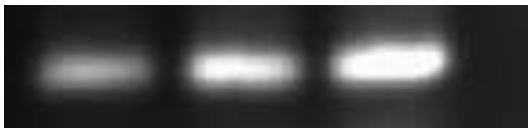


Figure 10: PAL gene expression in GIII non.SE- 42 days old callus, SE- 42 days and SE- 63 days old. (lane 1- GIII,42 days; lane 2- SE,42 days, lane 3- SE, 63 days)

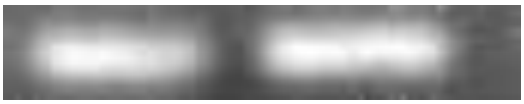


Figure 11: PPO *gene* expression in GIII and GIV 42 days old callus.(lane 1- GIII and lane 2- GIV)

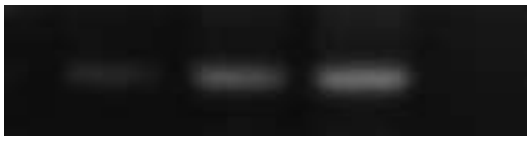


Figure 12:PPO *gene* expression in GIII non.SE- 42 days old callus, SE- 42 days and SE- 63 days old.(lane 1- GIII,42 days; lane 2- SE,42 days, lane 3- SE, 63 days)

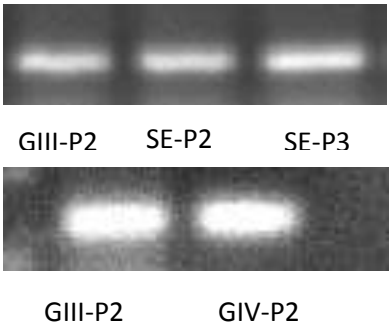


Figure 13: Beta actin as endogenous control.

Detection of some beneficial polyphenols in non-SE callus and SE callus

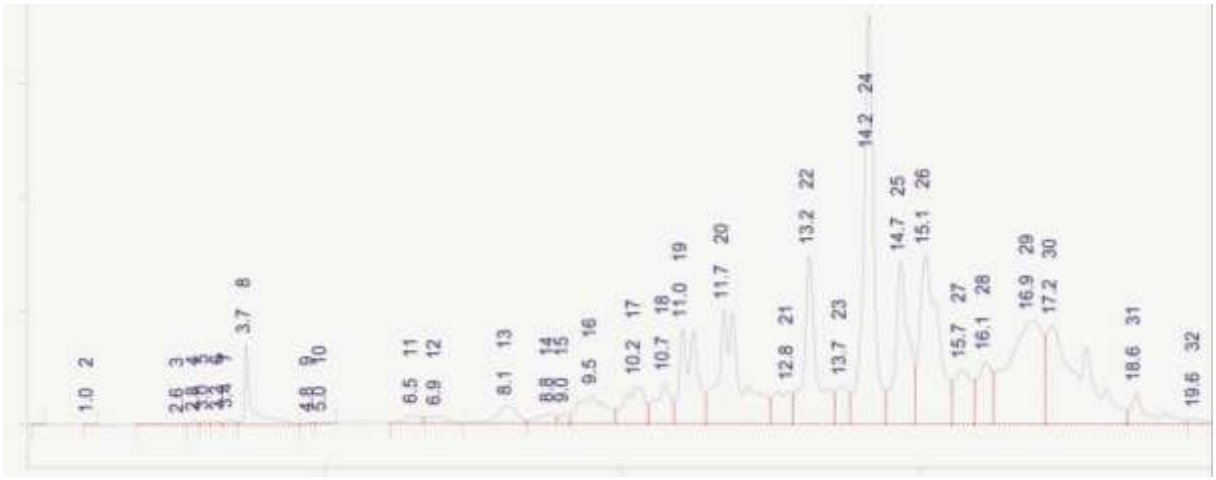


Figure 14: HPLC chromatogram of Non-SE (P2-GIII)

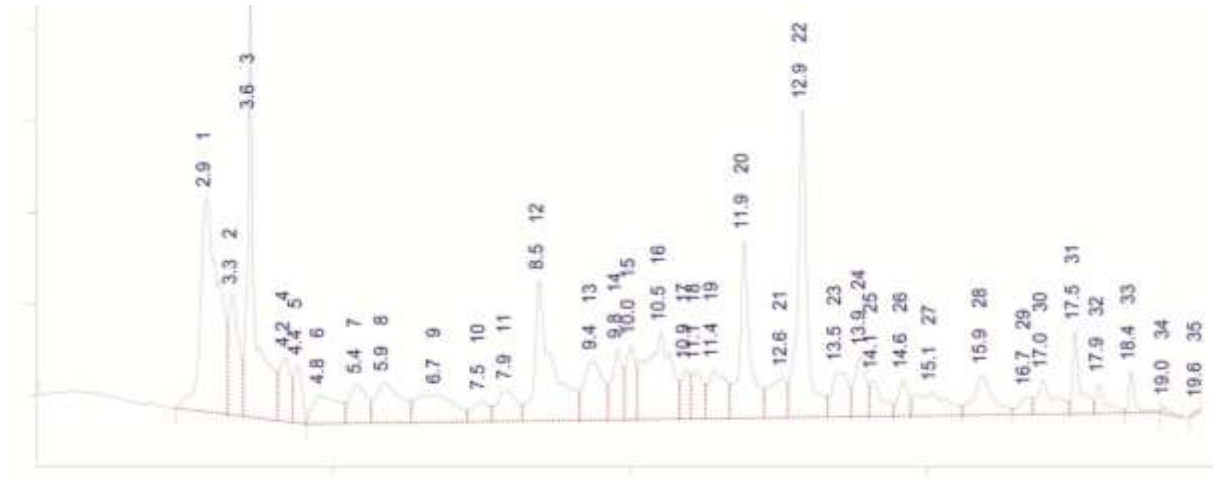


Figure 15: HPLC chromatogram of SE(63 days)

Conclusion

- Total polyphenol accumulation was highest in 63 days SE callus, Flavonoid like Rutin found to accumulate more in 63 days SE
- Some of the polyphenol got enhanced in SE callus, depicting some positive role in inducing SE in P.ovata.

Chapter 4: Chromium (VI) induced stress response in *Plantago ovata* Forsk. *in vitro*

Introduction

Heavy metals are natural constituent of soil but the pollution caused due to unregulated disposal induces stress both to plants and animals. Plants growing on such contaminated soil found to show several physiological changes to combat such heavy metal stress. Chromite is a natural form of Cr and found in ultramatic and serpentine rocks, in other compound forms as Crocoite ($PbCrO_4$), Tarapacite (K_2CrO_4), etc. [1].Chromium is also one of the heavy metal pollutants from various sources like steel industries, metal smelters, leather tanning, and emission from some industries and from pesticides and fertilizers[2,3,4,5]. An institute, works for heavy metal pollution control in many parts of the world, reported hexavalent chromium pollution in different industrial zone of India like Kanpur, Kolkata, Ranipet, Sukindra Valley and Vadodara, Gujarat [6].The soil of Surat, Gujarat also contained heavy metal pollutants; chromium presence was 305.2 mg/kg of soil, higher than the permissible limit [7]. Chromium is one of the 129^{th} prime pollutants and listed as harmful heavy metals. Inappropriate disposal of industrial waste containing chromium leads to its pollution in soil, and chromium (VI) is being very stable and more soluble than chromium (III) also causes groundwater pollution [8].

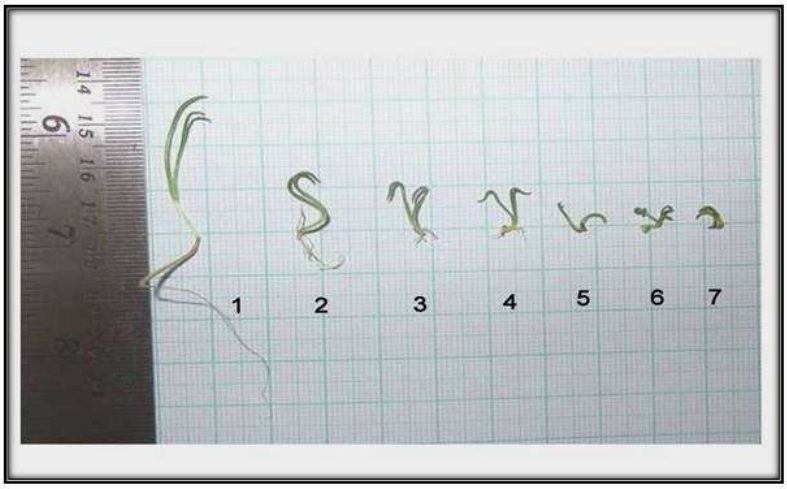
Many studies have been conducted in relation to the heavy metal tolerance of the genus *Plantago*. Serrano *et al.* [18] have corroborated the hyperaccumulation trait among *Plantago*. Several genera of *Plantago* shows hyperaccumulation of some heavy metal pollutants like Aluminium, Zinc, Copper, lead, etc [18].*Plantago arenaria* is tolerant to Cu, Cd, Ni and Zn [19]. Khan *et al.* [20] considered *P.ovata* as hyperaccumulator of lead as it can grow in soil with lead concentration up to 4000 μmol. Study of hexavalent chromium effects on *P.ovata* wasn't carried

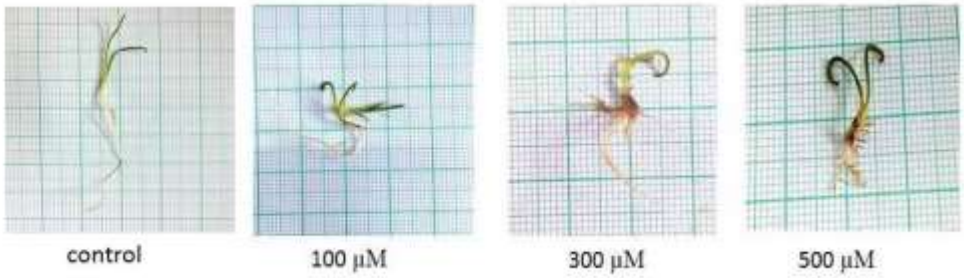
out yet. Effect of hexavalent chromium on *P.ovata* and its responsiveness towards the stress is being depicted in this study.

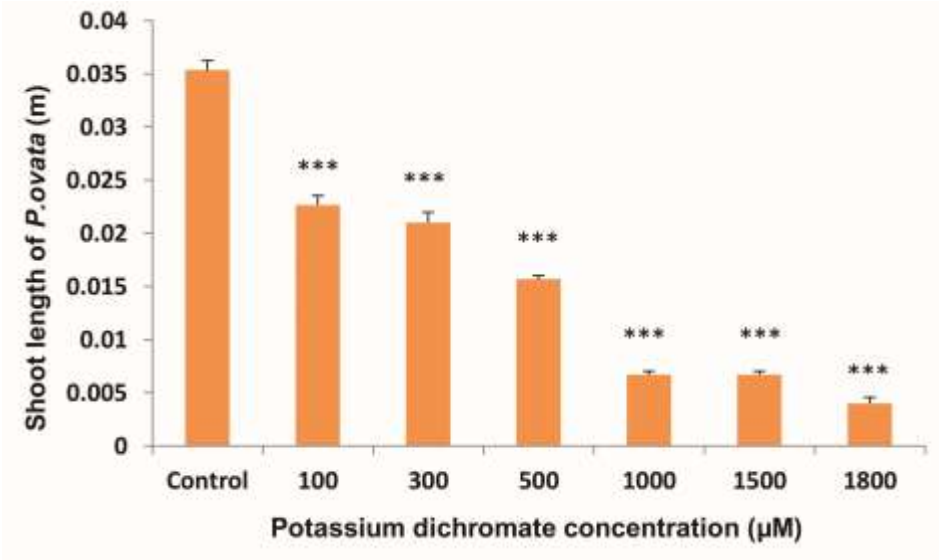
Results

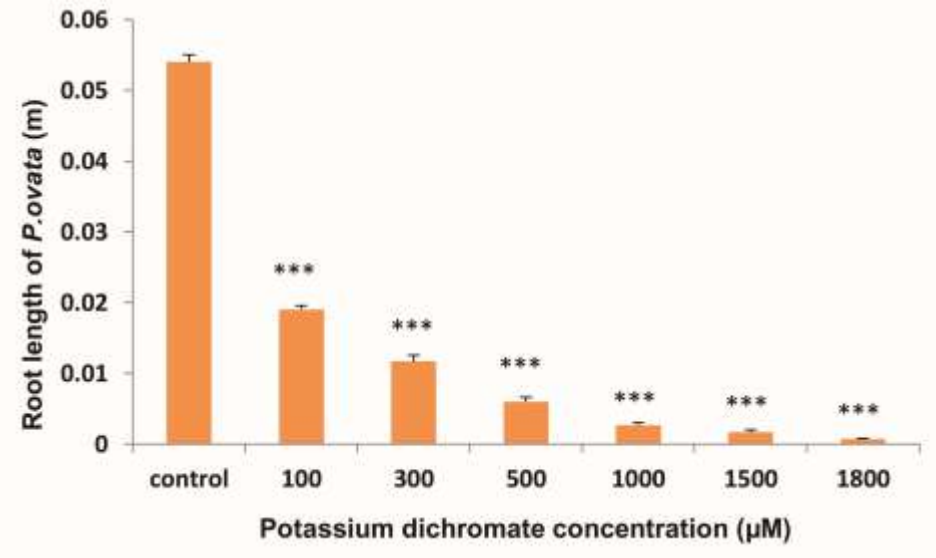
Morphological analysis

Morphological changes occurred in shoot and root length on exposure to chromium (VI) stress. Roots got more affected than shoot. Significant changes have occurred in a dose-dependent manner as compared to the control (**Fig 1**). Multiple root growth was seen in *P.ovata* seedlings with chromium stress (100 µM, 300 µM and 500 µM) (**Fig 2**). Though morphological changes due to chromium stress (VI) took place by affecting the shoot and root length (**Fig3**) and (**Fig 4**) respectively. *P.ovata* seedlings seemed quite tolerant in respect of germination rate and multiple root growth up to 500 µM.



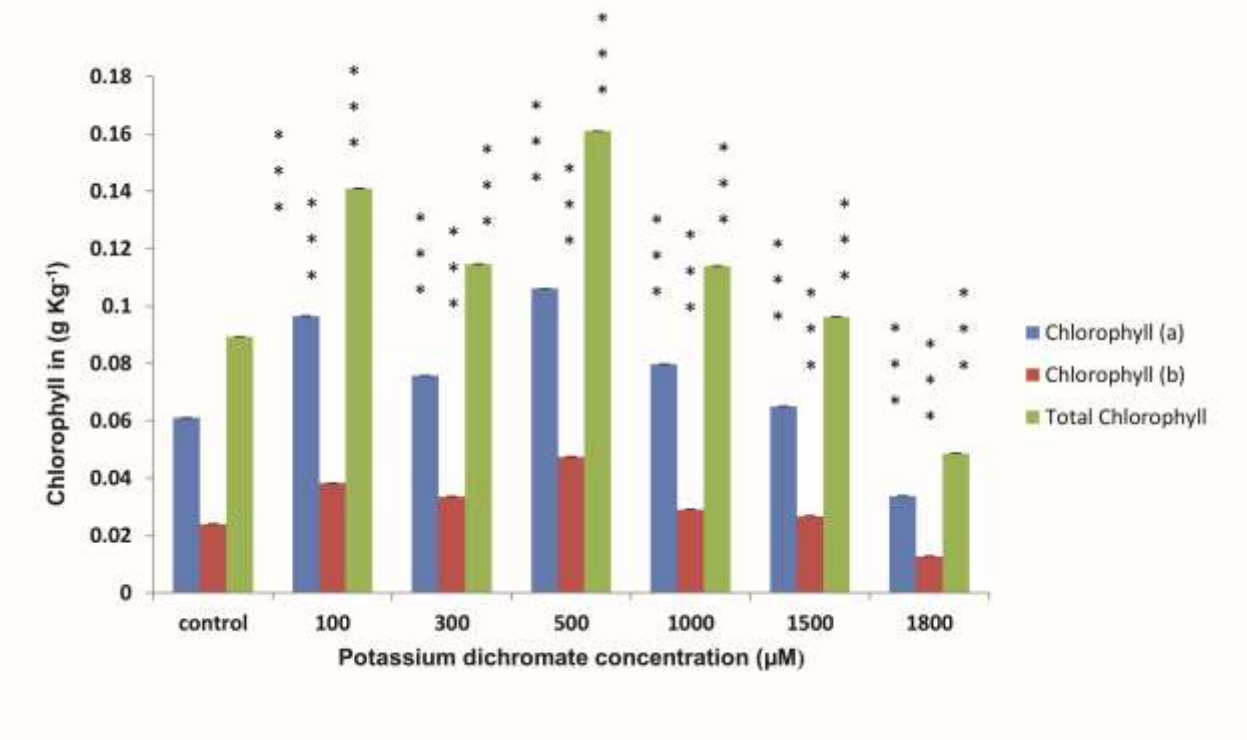

control
100 µM
300 µM
500 µM

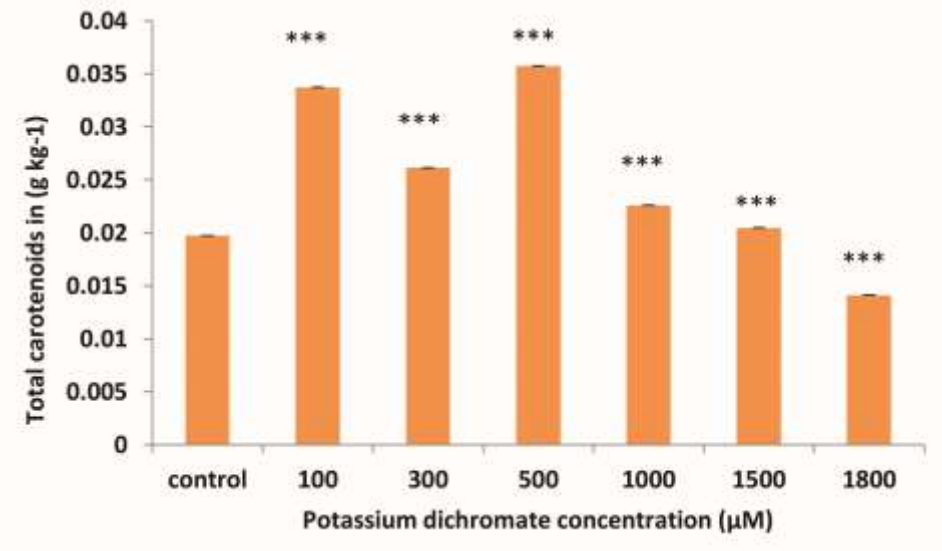

Shoot length of P.ovata (m)
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
***
***
***
***
***
***
Control
100
300
500
1000
1500
1800
Potassium dichromate concentration (µM)



Chlorophyll and carotenoid content

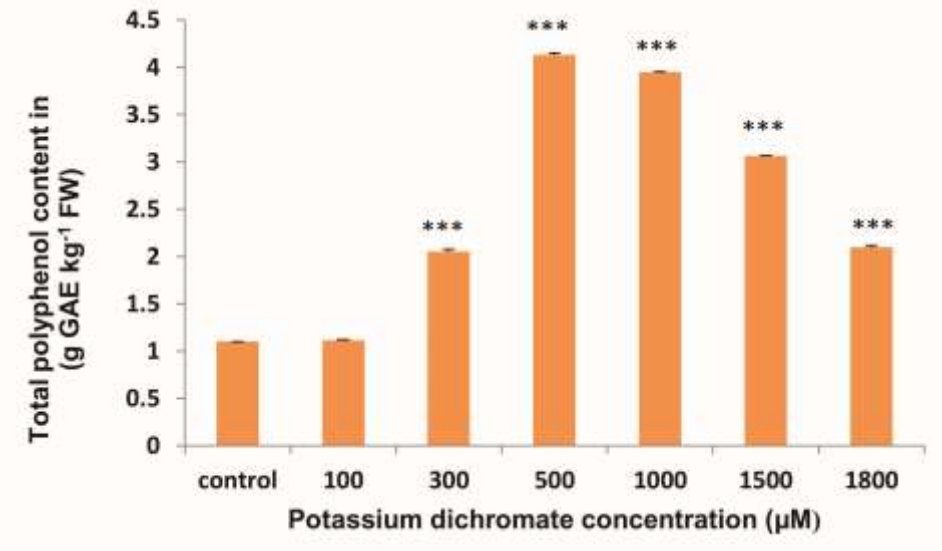
Chlorophyll (a, b and total chlorophyll) and carotenoid content increased significantly on Cr (VI) exposure up to 1500 µM concentration. The highest content was with 500 µM and lowest with 1800 µM doses **(Fig 5).** Carotenoid content followed the same trend as the chlorophyll content **(Fig 6)**





Total Polyphenol content

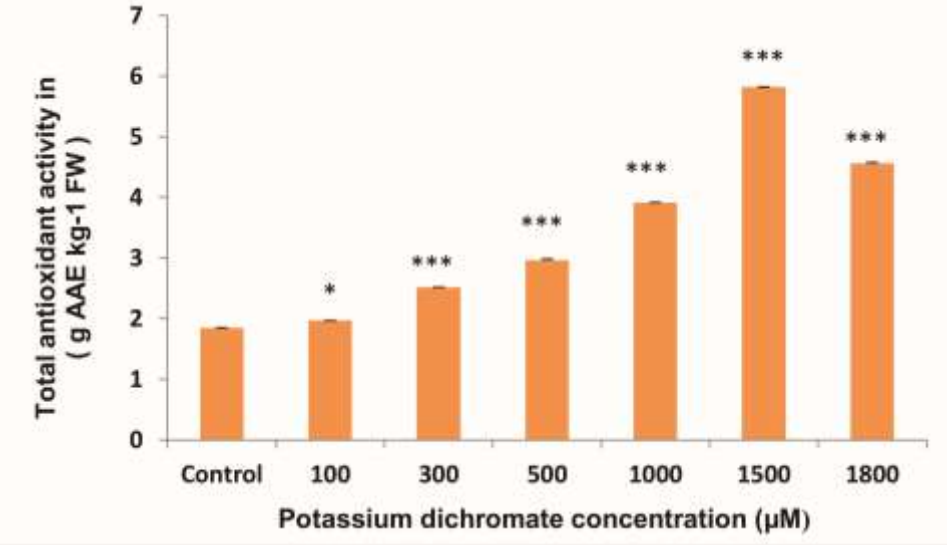
As discussed earlier exposure to heavy metals stress leads to increase in plant secondary metabolites and polyphenol accumulation as plant secondary metabolites got increased significantly in this study with chromium stress in a dose-dependent manner **(Fig 7)**. Total polyphenol content increased to about 3.75 times with 500 µM dose compared to the control, highest among the other doses.



Total Antioxidant activity

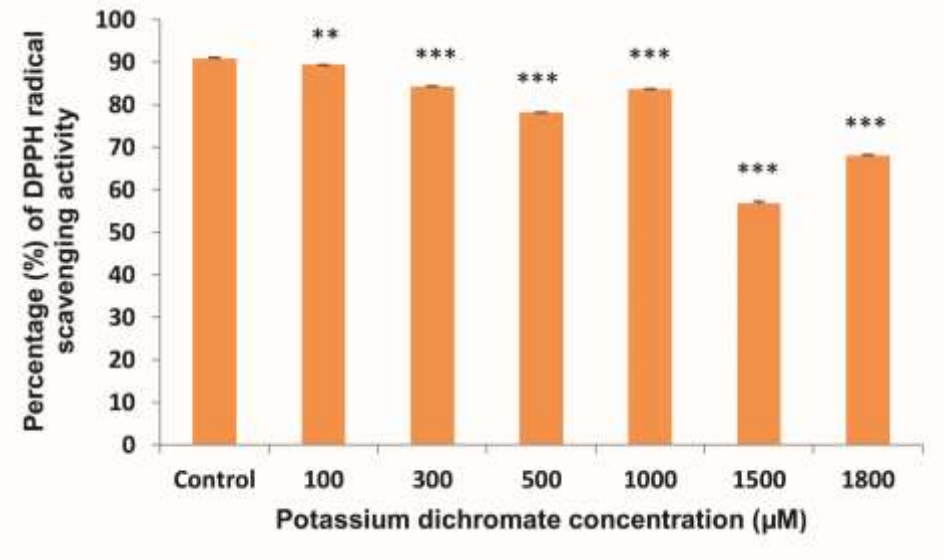
Increase in total antioxidant is a response of a plant to stress exposure and it protects the plant to survive by quenching ROS. In our experiment steady and significant increase in total antioxidant

activity occurred as compared to control with up to dose 1500 µM, then slightly the trend declined with the highest Cr dose in the experiment **(Fig 8)**



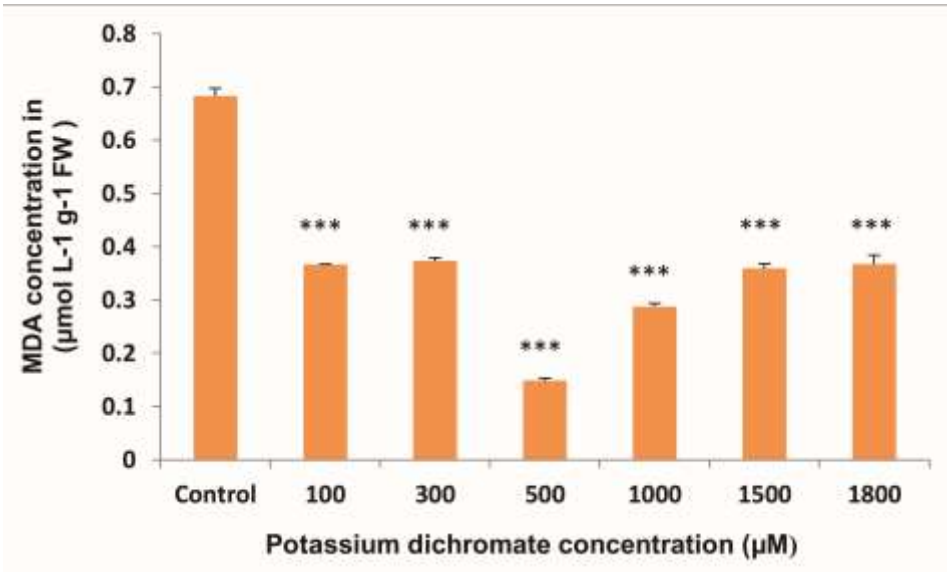
DPPH radical scavenging activity

Total antioxidant increased with the stress but unlikely, DPPH radical scavenging activity or the percentage of inhibition did not increase as compared to the control. The decrease was significant but the difference to that of control was little till 1000 µM of Cr (VI) stress **(Fig 9)** Percentage of inhibition was 90.81 in control plant and with Cr dose of 100 µM, 300 µM, 500 µM,1000 µM were 89.43, 84.31, 78.24 and 83.71 respectively. At concentration 1500 µM and 1800 µM the magnitude decreased sharply to 56.78% and 68.09% respectively.



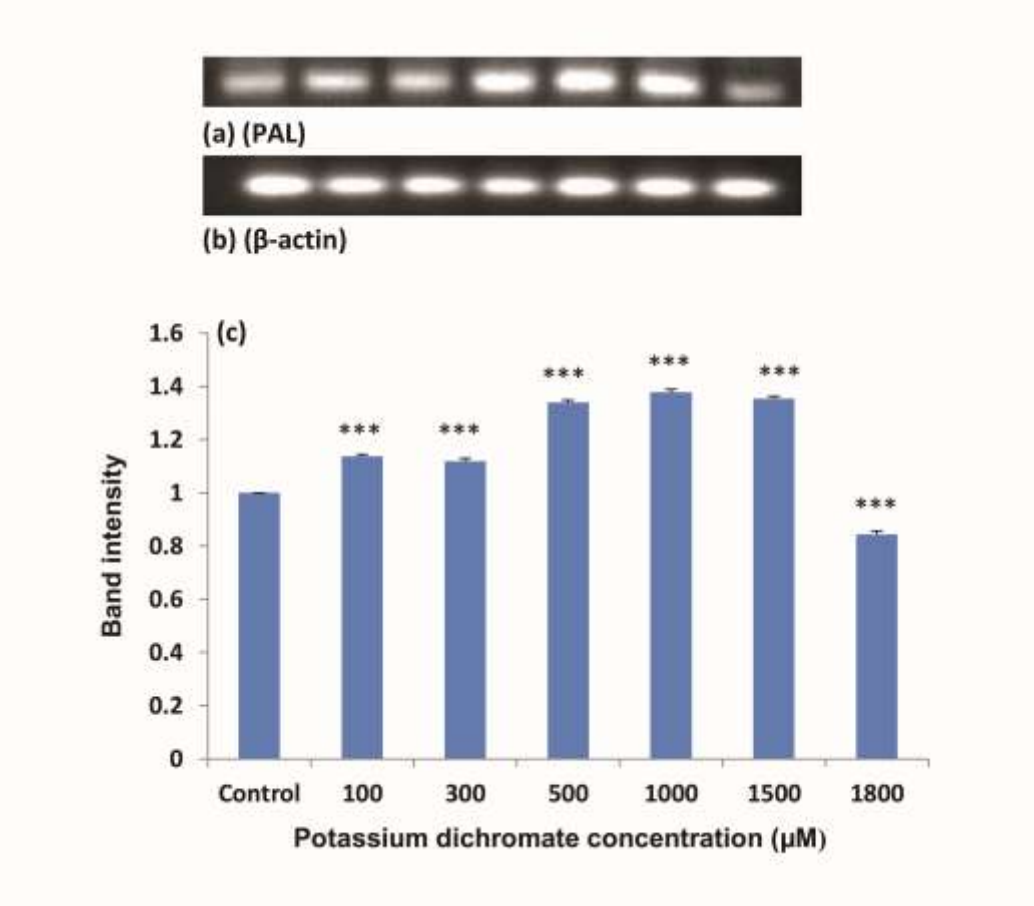
Lipid peroxidation

MDA content was significantly low with Cr stress, and the low MDA content proved lower lipid peroxidation. Plant showed its tolerant behavior and stress management to some extent. **(Fig 10)**



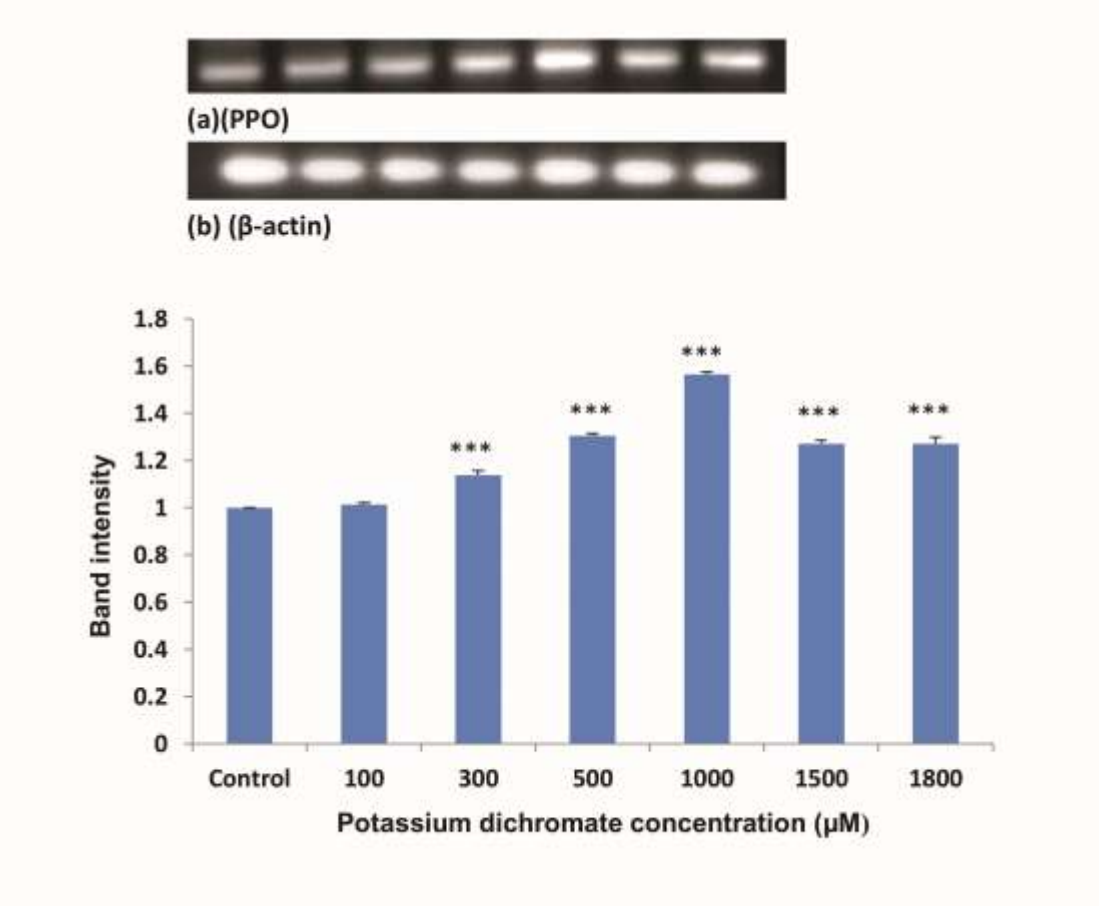
***PAL* gene expression analysis**

Phenyl-alanine ammonia lyase (*PAL*) gene upregulation was significant with stress exposure (hexavalent Cr). Band intensity showed significant rise with stress to that of control, highest increase in 1.377 fold intensity with 1000 µM Cr VI) dose **(Fig 11)**



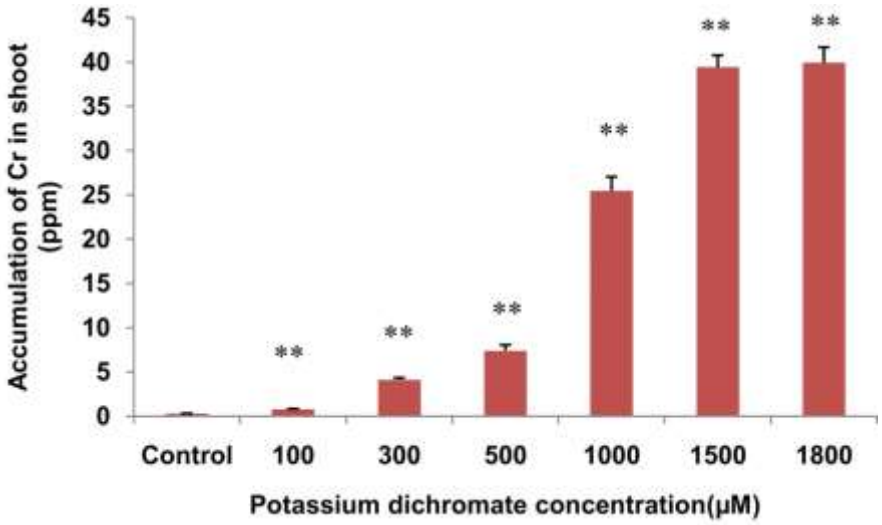
***PPO* gene expression analysis**

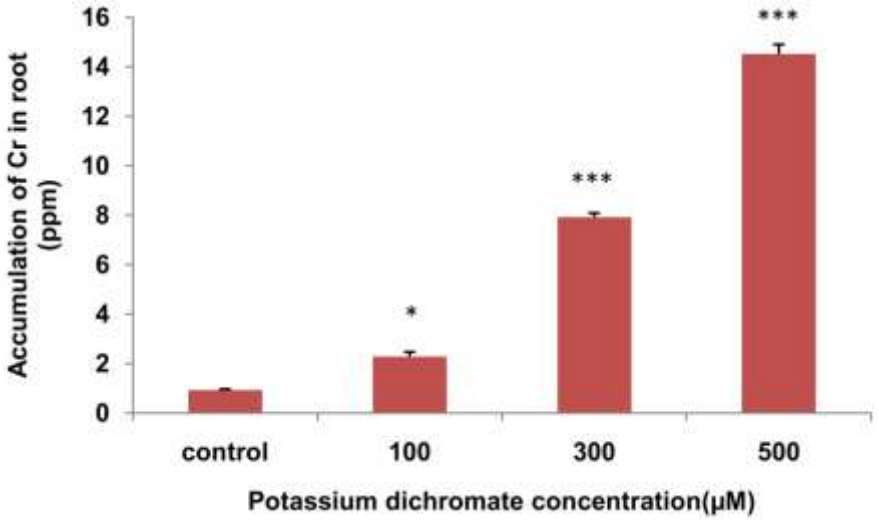
Polyphenol oxidase expression also got upregulated with stress **(Fig 12)** and being an antioxidative enzyme [2, 11] its upregulation might signify as a defensive response against the heavy metal toxins. Upregulation of expression was highest with 1000 μM Cr (VI), 1.56 fold increased as compared to the control.



Chromium accumulation detection by AAS.

Chromium accumulation in shoot and root of *P.ovata* increased with an increase of potassium dichromate concentration in the germination medium **(Fig 13 and Fig 14)** respectively. Control shoot with no potassium dichromate in the media showed chromium presence of about 0.27045ppm, with a dose of 100 µM (29.419 mg/L) in the germination medium, caused an increase of Cr accumulation by 2.88 fold. The accumulation of Cr increased by 147.55 fold in the shoot of *P.ovata*, highest in the experiment with 1800 µM dose (529.533 mg/L).In the root of control *P.ovata* seedlings; Cr presence was at an amount of 0.92815 ppm, comparatively more than in shoot region. Similarly as in shoot part; in the root also Cr accumulation got enhanced in a dose-dependent manner from low to high Cr concentration in the medium. Significant root growth of *P.ovata* seedlings took place with 0 µM , 100µM, 300µM and 500 µM Cr doses and not more than that, and the AAS results showed the Cr accumulation increased by 15.64 fold in root with 500 µM potassium dichromate as compared to the control *P.ovata* root.





Conclusion- Nature has its own way to deal with any harsh condition, be it any biotic or abiotic stresses. In our study on *P.ovata* with abiotic stress (hexavalent chromium) gave us a brief idea of its behavior of tolerance to some extent. Life always tries for its existence and is also built with such mechanisms. An increase of plant secondary metabolites, total antioxidant, overexpression of *PAL* and *PPO* gene in *P.ovata* and Cr uptake by the plant to some extent, depicted its survival strategy and tolerant response towards the Cr stress *in vitro.*

Structure prediction and homology modeling of partial sequence of PPO

Partial sequence of Polyphenol oxidase (*PPO*) of about 422bpcdswas sequenced for structural and homology analysis. Partial cds of the PPO gene translated into a 140 amino acid long sequence by *in silico* analysis [GenBank: KM192264.1]. SignalIP prediction showed an absence of signal peptide in (figure: 8a), in the partial sequence of the PPO gene. TMpred software prediction predicted the model showing one strong trans-membrane helices of 22 amino acids between 109 to 130 amino acid regions showed in (Figure: 8c). The Protscale hydropathicity score (Figure: 8b), corroborate the maximum hydrophobicity between the positions 122 to 124, 1.378 as maximum score. Swiss model analysis showed the template based homology with 33 templates. Maximum of those templates were named as Polyphenol oxidase, Catechol oxidase, tyrosinase. Polyphenol oxidase is also known by other names like Catechol oxidase, Tyrosinase, etc [Yoruk and Marshall, 2003]. So the result predicted the homology with other Polyphenol oxidase of other plant species. Out of the 33 template, the maximum homology is with Catechol oxidase of sweet potato. The built model of the 3D structure of PPO (Figure: 8d) analysis showed it as monomer protein with 50.36% similarity with sweet potato 3D structure.

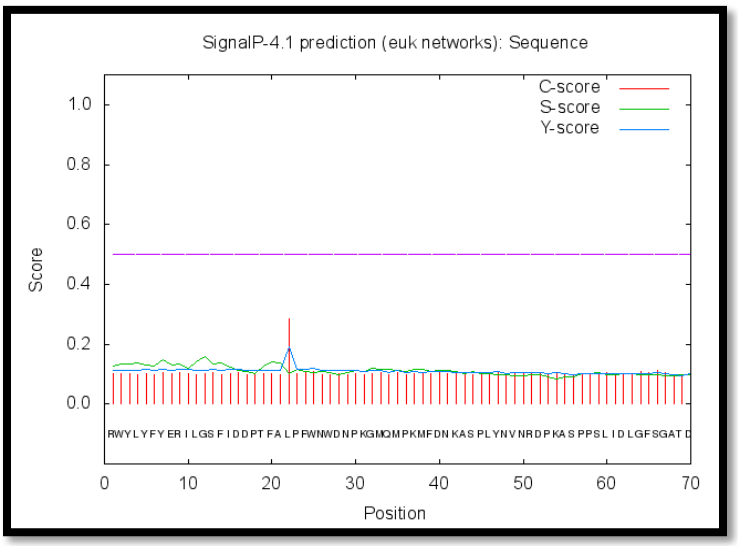


Figure 8: (a)

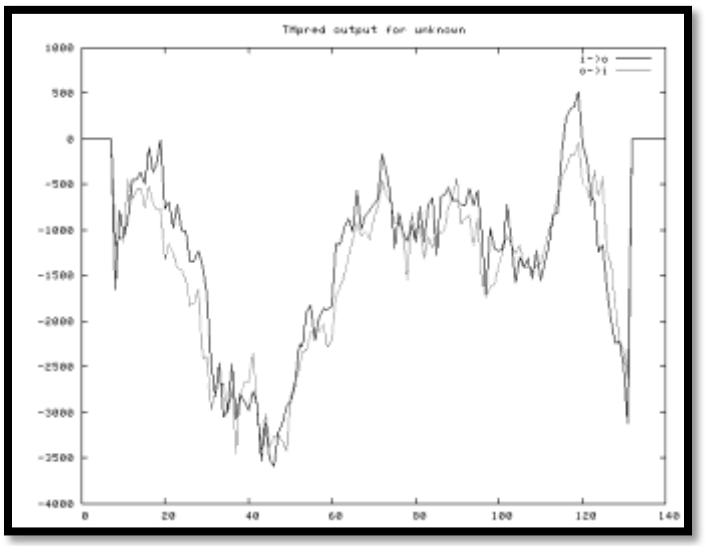


Figure 8: (b)

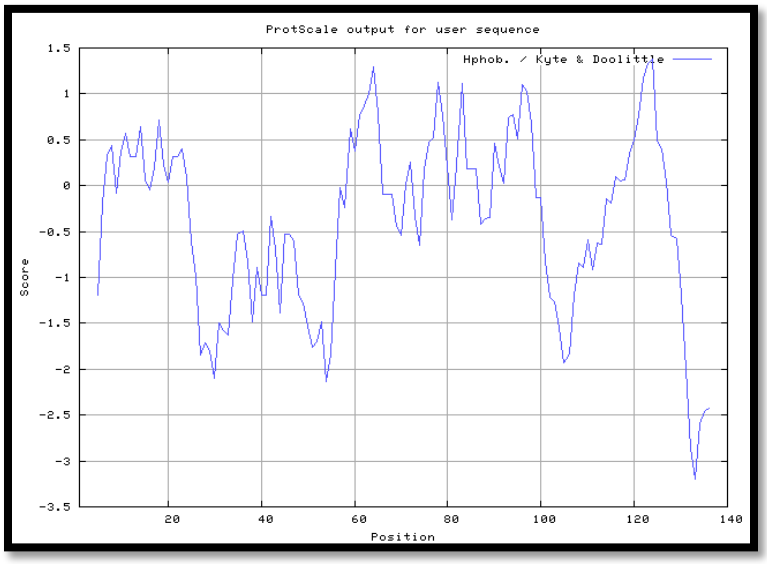


Figure 8: (c)

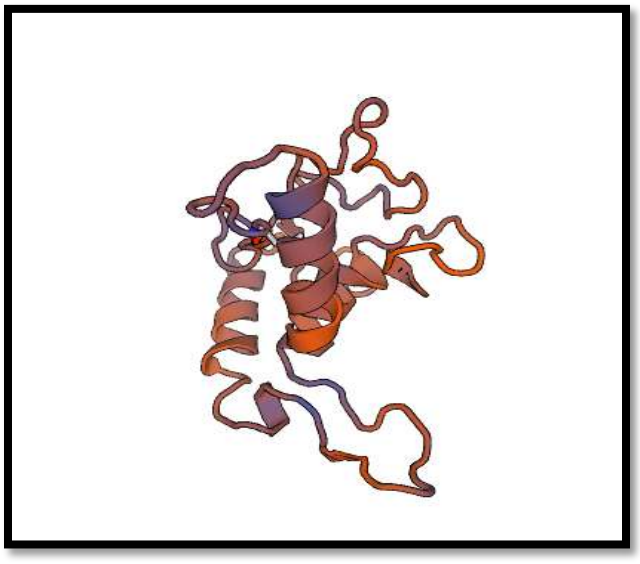


Figure 8: (d)

Figure 8: (a) Prediction of signal peptide by SignalP; **(b)** Prediction of transmembrane domain by TMpred tool; X-axis: Number of amino acids; Y-axis: Hydrophobicity score; **(c)** The predicted three dimensional model of *P. ovata* PPO protein; **(d)** Hydropathy plot showing the hydropathy score of the amino acids of predicted protein

Conclusion-

- The objective of the study was to enhance polyphenol accumulation through tissue culture technique.
- Different hormone combinations were used to set up callogenesis.
- The result depicted that the combination of hormones with kinetin concentration higher (group B and C) compare to other combinations accumulated more polyphenols.
- The result was further verified with another experimental setup of four groups with ascending kinetin concentration from Group I-IV and with fixed 2,4-D concentration .
- This study proved the positive impact of Kinetin in accumulating polyphenols and suggested its application commercially as in optimum doses less browning of callus tissue occurred referring down-regulation of *PPO* gene expression whereas *PAL* gene expression was up-regulated compare to the control group I.
- Somatic embryos were cultured with specific hormones and Polyphenols accumulation was analyzed.

- *P.ovata* SE of 63 days showed the highet accumulation of total polyphenols compare to non-Se and 48 days SE.
- *P.ovata* is an important medicinal plant and many literature depicted hyper accumulating characteristics of its Genus to some specific heavy metal.
- The two concern gene of the study PPO and PAL are stressed induced gene and plant secondary metabolities like polyphenols play important role in managing plant health in presence of abiotic stress.
- Responsiveness of *P.ovata* in presence of chromium stress was studied. Hexavalent chromium is a pollutant and also present in region where Plantago grows to great extent like in Gujrat and other parts of India.
- The study depicted the tolerance level of P.ovata to the Cr stress and how plant secondary metabolities and other biochemical attributes get regulated according to the level of stress to make the plant tolerant to withstand the stress.
- Conclusively Kinetin played an important role in enhancing polyphenol content. At optimum level adequate amount of polyphenols can be enhanced. Significant level of polyphenol was also increased in 63 days SE. inducing SE was also a good option for polyphenol enhancement and plant propagation as well.

Reference

Akula R & Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling & Behavior.2011; 6:1720-1731

Alderson PG and Nagarajan A (2012) Effect of growth hormones on callus induction of *Sauropus androgynous* (sweet shoot).Annals of Biological Research 3:4668-4674

Alla MMN, Younis ME, Shihaby OA, Bastawisy ZM (2002). Kinetin regulation of growth and secondary metabolism in water logging and salinity treated *Vigna sinesis* and *Zea mays*. Acta Physiologiae Plantarum 24:19-27.

AnastasiaE, Ginnakoula, Ilias F. IIlias, Jelena J. Dragisic Maksimovic, Vuk M. Maksimovic and Branka D. Zivanovic(2012). The effect of Plant growth regulators on growth, yield, and phenolic profile of Lentil plants. *Journal of Food Composition and Analysis*;28:46-53

Angelova Y, Petkova S, Zozikova E, Kotseva E,Iliev L (2001).Effect of Kinetin and 4PU-30 on the growth and the content of Polyphenols in tobacco Callus tissue. *Bulg.J.Plant Phisiol*; 27(1-2):36-42.

Arts ICW, Hollman PCH(2005). Polyphenols and disease risk in epidemiologic studies.*Am J Clin Nutr*;81:317-25.

Asati A, Pichhode M, Nikhil K. Effect of heavy metals on plants: an overview. International Journal of Application or Innovation in Engineering & Management. 2016;5(3):56-61.

Baker AJ, Walker P. Physiological responses of plants to heavy metals and the quantification of tolerance and toxicity. Chemical Speciation & Bioavailability. 1989 Mar 1;1(1):7-17.

Barcel´O.J., Poschenrieder, C. and Guns´e, J. Effect of chromium (VI) on mineral element composition of bush beans. Journal of Plant Nutrition. 1985.8:211-217.

Barciszewski J,Siboska G, Rattan SIS, Clark BFC (2000) Occurrence,biosynthesis and properties of kinetin(N^6- furfuryladenine)

Baslam M, Garmendia I, Goicoeche N. Enhanced accumulation of vitamins, nutraceuticals and minerals in lettuces associated with arbuscular mycorrhizal fungi (AMF): A question of interest for both vegetables and humans. Agriculture.2013; 3:188-2019.

Bevan M and Northcote DH (1979) The interaction of auxin and cytokinin in the induction of Phenylalanine Ammonia-Lyase in suspension culture of *Phaseolus vulgaris*. Planta 147:77-81

Blacksmithinstitute.New York.1999.

Boonyapookana B, Upatham ES, Kruatrachue M, Pokethitiyook P, Singhakaew S.Phytoaccumulation and phytotoxicity of cadmium and chromium in duckweed Wolffia globosa. Int J Phytoremed. 2002;4:87–100.

Brand-Williams W, Cuvelier ME and Berset C.Use of a free radical method to evaluate antioxidant activity. Lebensm-Wiss Technol.1995;28:25–30.

Chang J, Luo J,He G (2009) Regulation of polyphenols accumulation by combined overexpression/ silencing key enzymes of phenylpropanoid pathway. Acta Biochim Biophys Sin 41: 123–130

Chen L, Mehta A,Berenbaum M, Zangeri AR,Engeseth NJ (2000) Honeys from different floral sourses as inhibitors of enzymatic browning in fruit and vegetable homogenates. J.Agric.Food Chem 48:4997-5000

Corradi MG, Bianchi A, Albasini A. Chromium toxicity in salvia sclarea-I. effects of hexavalent chromium on seed germination and seedling development.Environmental and Experimental Botany.1993;33(3):405-413

Das Pal M and Sen Raychaudhuri S. Enhanced development of somatic embryos of *Plantago ovata* Forsk. by additives. In Vitro Cell Dev Biol.2001;37:568–571.

Devi Chinmayee M, Anu M, Mahesh B, Mary S, Mini I, Swapna TS. A comparative study of heavy metal accumulation and antioxidant responses in Jatropha curcas. IOSR Journal of Environmental Science, Toxicology and Food Technology. 2014;8(7):58-67.

Dhar MK, Kaul S, Sareen S, Koul AK (2005) *Plantago ovata*: genetic diversity, cultivation, utilization and chemistry. Plant genetic resources 3(2):252-263

Edward K, JohnstoneC and ThompsonC.A simple and rapid method for the presentation of plant genomic DNA for PCR analysis.Nucleic Acids Res ;19:1349(1991)

Emamverdian A, Ding Y, Mokhberdoran F, Xie Y. Heavy metal stress and some mechanisms of plant defense response. The Scientific World Journal. 2015 Jan 26;2015.

Goud PB and Kaechole MS (2012) Antioxidant enzyme changes in Neem, Pigeonpea and Mulberry leaves in two stages of maturity. Plant Signaling and Behavior 7: 1258- 1262

Grof BA, Milbury PE, Blumberg JB (2005) Flavanols, flavonones,flavanones and human health: epidemiological evidence. J Med Food 8: 281-290

Hadif WM, Rahim SA, Sahid I, Rahman A, Ibrahim I. Influence of chromium metal on chlorophyll content in leaves of paddy *Oryza sativa* L. International Journal of Chemical Sciences. 2015;13(3)

Hamayun M, Hussain A, Khan S A,Irshad M,Khan A L, Waqas M, Shahzad R, Iqbal A, Ullah N, Rehman G, Kim H Y , Lee I J (2015) Kinetin modulates physio-hormonal attributes and isoflavone contents of Soybean grown under salinity stress.

Heath RL and Packer L.Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys.1968;125:189–198.

Heqiang L,Ping C,Hong Z,Cuicui X and Hongfel L. Effect of Kinetin on quality and harvest date of Loquat fruit.Afr.J.Agric.Res; 7(10):1577-1583.(2012)

HolderbaumD.F,Kon T,KudoT,GuenM.P(2010).Enzymatic browning, Polyphenol Oxidase Activity & Polyphenolsin four Apple cultivars : Dynamics during fruit development. *Hort Science*; 45(8): 1150-1154.

Ibrahim MH, Kong YC, Zain NAM. Effect of Cadmium and Copper Exposure on Growth, Secondary Metabolites and Antioxidant Activity in the Medicinal Plant Sambung Nyawa (Gynura procumbens (Lour.) Merr).Molecules.2017; 22:1623.doi:10.3390/molecules22101623

Jong F D, Hanley S J ,Beale M H, Karp A (2015) Characterisation of the willow phenylalanine ammonia-lyase (PAL) gene family reveals expression differences compared with poplar. Phytochemistry 117 :90– 97.

Karolak I G, Kuz´ma L, Wysokinska H (2015) The effect of cytokinins on shoot proliferation, secondary metabolite production and antioxidant potential in shoot cultures of *Scutellariaalpina.* Plant Cell Tiss Organ Cult 122:699–708..

Khan ZI, Kashaf S, Ahmad K, Akram NA, Ashraf M, Mahmood SU, Sohail M, Bashir H, Mehmood N. Metal uptake by psyllium (*Plantago ovata* L.) treated with lead (Pb) under semi-arid conditions. Legume Research-An International Journal. 2017 Apr 1;40(2):277-81.

Kouakou TH, Waffo-Téguo P, Kouadio YJ, Valls J, Richard T, Decendit A, Mérillon JM. Phenolic compounds and somatic embryogenesis in cotton (Gossypium hirsutum L.). Plant cell, tissue and organ culture. 2007 Jul 1;90(1):25-9.

Kováčik J, Babula P, Klejdus B, Hedbavny J. Chromium uptake and consequences for metabolism and oxidative stress in Chamomile plants. J. Agric. Food Chem. 2013; 61:7864−7873

Krishna, A.K. & Govil, P.K. Soil contamination due to heavy metals from an industrial area of Surat, Gujarat, Western India. Environ Monit Assess (2007) 124: 263.

Kumar N1, Shibata D, Helm J, Coppola D, Malafa M (2007). Green tea polyphenols in the prevention of colon cancer.*Front Biosci*.;12:2309-15.

Le BC, Le QJM, Sanoner P, Drilleau JF, Guyot S (2004) Inhibition of applepolyphenol oxidase activity by procyanidins and polyphenol oxidation products. J.Agric.Food Chem 52:122-130

Lee C.Y, KaganV,JaworskiA.W,Brown S.K (1990).Enzymatic Browning In Relation to Phenolic Compounds And Polyphenoloxidase Activity Among Various Peach Cultivars.*J.Agric.FoodChem*; 38:99-101.

Li C, Li D, Li J, Shao F, Lu S (2017) Characterization of the polyphenol oxidase gene family reveals a novel micro RNA involved in post transcriptional regulation of PPOs in *Salvia miltiorrhiza*. Scientific reports (7): 44622.

Lichtenthaler HK.Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol .1987;148:350–382

Lin JY and Tang CY (2007) Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem 101:140– 147

Malmir HA. The relations between phenylalanine–ammonia lyase,glutathione-s-transferase activities and the concentrations of total tannins, phytochelatins, glutathione, and peroxidation in two cultivars of Sorghum (sorghum bicolor (L) moench)exposed to aluminum. Agric Res.2012;1(3):240–250

Mohanty M, Patra HK. Attenuation of chromium toxicity by bioremediation technology. Reviews of Environmental Contamination and Toxicology.2011;210:1-34

Moumou Y, Vasseur J, Trotin F, Dubois J(1992).Catechin production by callus culture of Fagopyrumesculentum. Phytochemistry.31:1239-1241

NagaiN,KojimaY,ShimosakaM &OkazakiM(1988) .Effects Of Kinetin on L-Phenylalanine Ammonia-Lyase Activity in Tobacco Cell Culture.*Agric.Biol.Chem*;52(10):2617-2619

Okem, A, StirkWA,Street RA, Southway C, Finnie JF, Van Staden J. Effects of Cd and Al stress on secondary metabolites, antioxidant and antibacterial activity of Hypoxis hemerocallidea Fisch. & C.A. Mey. Plant Physiol. Biochem. 2015;97:147–155

Oliveira H. Chromium as an environmental pollutant: insights on induced plant toxicity. Journal of botany. 2012 May 20;2012.

Pandey KB and Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity 2:270-278

Peralta JR, Gardea-Torresdey JL, Tiemann KJ, Gomez E, Arteaga S, Rascon E, Parsons JG. Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (Medicago sativa L.). Bulletin of Environmental Contamination and toxicology. 2001 Jun 24;66(6):727-34.

Prieto P, Pineda M and Aguilar M.Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitaminE. Anal Biochem .1999;269:337–341

Rai V & Mehrotra S. Chromium-induced changes in ultramorphology and secondary metabolites of Phyllanthus amarus Schum & Thonn. – an hepatoprotective plant. Environ Monit Assess .2008;147:307–315

Rai V, Tandon PK, Khatoon S. Effect of chromium on antioxidant potential of Catharanthus roseus varieties and production of their anticancer alkaloids: vincristine and vinblastine. BioMed research international. 2014 Mar 10;2014.

Rai V, Vajpayee P, Singh SN, Mehrotra S. Effect of chromium accumulation on photosynthetic pigments, oxidative stress defense system, nitrate reduction, proline level and eugenol content of *Ocimum tenuiflorum* L. Plant Science.2004;167: 1159–1169

Ramakrishna A and Ravishankar GA (2017) Inflence of abiotic stress signals on secondary metabolites in plants. Plant signaling and Behavior 6: 1720-1731.

Remon E, BOuchardon JL, Faure O. Multi-tolerance to heavy metals in *Plantago arenaria* Waldst.& Kit.: Adaptive versus constitutive characters.Chemosphere.2007;69:41-47

Ross IA (2005) Medicinal plants of the world. Human Press Inc,Totowa,New Jersey 3: 419-459.

Sangwan P, Kumar V, and Joshi UN. Effect of chromium (VI) toxicity on enzymes of nitrogen metabolism in Clusterbean (*Cyamopsis tetragonoloba* L). Enzyme Research.2014

Scalbert A, Manach C, Morand C, Remesy C(2005).Dietary Polyphenols and The prevention Of Diseases.*Crit Rev Food Sci Nutr*;45:287-306.

Serrano HC, Cotrim H, Pinto MJ, Loucao MAM.Metal hyperaccumulation patterns within *Plantago* phylogeny(Plantaginaceae).Plant Soil.2017;411:227-241.

Sestak Z, Catský J, Jarvis PG. Plant photosynthetic production. Manual of methods. Plant photosynthetic production. Manual of methods.. 1971.

Shah SH (2011) Kinetin improves photosysnthetic and antioxidant responses of *Nigella sativa* to counteract salt stress. Russian Journal of Plant Physiology 58:454-459.

Shah, R. R., K. V. Subbaiah, A. R. Mehta, 1976. Hormonal effect on polyphenol accumulation in Cassia tissues cultured in vitro. Can. J. Bot., 54, 1240–1245.

Shanker AK, Cervantes C, Loza-Tavera H, Avudainayagam S. Chromium toxicity in plants. Environment International.2005;31:739– 753

Sharma P, Bihari V, Agarwal SK, Verma V, Kesavachandran CN, Pangtey BS, Mathur N, Singh KP, Srivastava M, Goel SK. Groundwater Contaminated with Hexavalent Chromium [Cr (VI)]: A Health Survey and Clinical Examination of Community Inhabitants (Kanpur, India). PLOS ONE.2012; 7(10): e47877

Sharma YK and Daris KR (1994) Ozone induced expression of stress related genes in *Arabidopsis thaliana*.Plant Physiol 105:1089-1096.

Shukla OP, Dubey S, Rai UN. Preferential accumulation of cadmium and chromium: Toxicity in *Bacopa monnieri* L. under mixed metal treatments. Bull. Environ. Contam. Toxicol. 2007; 78:252–257

Shyamali S and Kazumi H (2007) Synergistic effect of kinetin and benzyl adenine improves the regeneration of cotyledon explants of Bottle gourd (*Lagenaria siceraria*) on ethylene production. Advances in plant ethylene research.7:153-161

Siaka M, Owens CM, Birch GF. Evaluation of some digestion methods for the determination of heavy metals in sediment samples by flame-AAS. Analytical letters. 1998 Feb 1; 31(4):703-18.

Singh B (2007) Psyllium as therapeutic and drug delivery agent. International Journal of Pharmaceutics 334:1-4

Singleton VL, Orthofer R and Lamuela-Raventos RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Methods Enzymol 299:152–178

Sinha V, Pakshirajan K, Chaturvedi R. Chromium tolerance, bioaccumulation and localization in plants: An overview. Journal of environmental management. 2018 Jan 15;206:715-30.

Spencer JP,Abd El Mohsen MM,MinihaneAM,Mathers JC(2008).Biomarkers Of The Intake Of Dietary Polyphenols: Strength,Limitations and Application In Nutrition Research.*Br JNutr*;99:12-22.

Sullivan ML. Beyond brown: polyphenol oxidases as enzymes of plant specialized metabolism. Frontiers in plant science. 2015 Jan 14;5:783.

Talukder P, Talapatra S, Ghoshal N, Sen Raychaudhuri S (2016) Antioxidant activity and high-performance liquid chromatographic analysis of phenolic compounds during *in vitro* callus culture of *Plantago ovata* Forsk and effect of exogenous additives on accumulation of phenolic compounds. J Sci Food Agric. 96: 232-244

Talukder P,Talapatra S, Ghoshal N and Sen Raychaudhuri S (2015). Antioxidant activity and high-performance liquid chromatographic analysis of Phenolic compounds during *in-vitro* callus culture of

*Plantagoovata*Forsk.and effect of exogenous additives on accumulation of phenolic compounds. *J Sci Food Agric*;

Tangney C and E.Rasmussen.H (2013). Polyphenols,Inflammation& Cardiovascular Disease.*NIH-PA*;15(5):324.

Tounekti T, Hernandez I, Muller M, Kemiza H, Bosch SM (2011) Kinetin application alleviate salt stress and improve the antioxidant compositionof leaf extract in *Salvia officinalis*. Plant Physiology and Biochemistry 49: 1165-1176

Traber MG and Atkinson J(2007) Vitamin E, antioxidant and nothing more. Free Radic Biol Med 43: 4-15

Uysal U1, Seremet S, Lamping JW, Adams JM, Liu DY, Swerdlow RH, Aires DJ(2013). Consumption of polyphenol plants may slow aging and associated diseases. *Current Pharmaceutical Design*; 19:6094-6111.

Vajpayee P, Tripathi RD, Rai UN, Ali MB, Singh SN. Chromium (VI) accumulation reduces chlorophyll biosynthesis, nitrate reductase activity and protein content in *Nymphaea alba* L. Chemosphere.2000;41: 1075-1082

Vamos-Vigyazo L(1981) Polyphenol oxidase and peroxidase in fruits and vegetables. Food Sci Nutr 15: 49- 127

Vasilaki A.T., McMillan D.C. (2011) Lipid Peroxidation. In: Schwab M. (eds) Encyclopedia of Cancer. Springer, Berlin, Heidelberg

Vogt T (2010) Phenylpropanoid biosynthesis. Mol. Plant 3: 2–20

WHO Chromium, Environmental Health Criteria 61.World Health Organization, Geneva (1988).

Wu J, Zhang X, Nie Y, Jin S,Liang S (2004) Factor affecting somatic embryogeneis and plant regeneration from a range of recalcitrant genotypes of Chinese cottons (Gossypium hirsutum L.). In Vitro Cell Biol 40:371–375

Yadav SK. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. South African Journal of Botany.2010; 76: 167–179.

Yoruk R and Marshall.R Maurice(2003).Physicochemical Properties and Function of Plant Polyphenol Oxidase: A Review. *Journal of Food Biochemistry* ; 27:261-422

Zagoskina N V, Zaprometov M N (1983) Effect of kinetin on formation of phenol compounds in the long-passaged tea plant tissue culture. Physiology and biochemistry of cultivated plants 15 : 250–253

Zhang XH, Liu J, Huang HT, Chen J, Zhu YN, Wang DQ. Chromium accumulation by the hyperaccumulator plant Leersia hexandra Swartz. Chemosphere. 2007 Apr 1;67(6):1138-43.

Publications and Gene Bank Submission

- **Kundu D and Talukder P, Sen Raychaudhuri S.(2018). *In vitro* Biosynthesis of Polyphenols in Presence of Elicitors and Upregulation of Genes of Phenylpropanoid Pathway in *Plantago ovata*. In Book: Studies on Natural product chemistry. Edited by Attaur Rahamen. Elsevier publications. (Accepted for publication)**
- **Kundu D, Sen Raychaudhuri S.(2018). Chromium (VI) induced stress response in *Plantago ovata* Forsk. *in vitro.* Genes and Environment (BMC). (Revision).**
- **Kundu D and Sen Raychaudhuri S .Gene Bank submission :*Plantago ovata* cultivar CIMAP chloroplast polyphenol oxidase mRNA, partial cds; nuclear gene for chloroplast product. GenBank: KM192264.1.**

Posters and oral presentations

- **Poster Presentation**

  Effect of Kinetin in Polyphenol Accumulation and expression of Polyphenol oxidase gene in *Plantago ovata* Forsk *in vitro.*- Recent advances in Research and Development in medicinal and aromatic plants- a country scenario at State Forest Research Institute (SFRI).

  Role of Kinetin in Accumulation of Polyphenols and expression of Polyphenol oxidase gene (*PPO*) in *Plantago ovata* Forsk *in vitro*-Exploring Biological Systems: Cell to Organism" (EBS-2016)

  Chromium (VI) induced stress response in *Plantago ovata* Forsk. *in vitro* .- All India Congress of Cytology and Genetics.

- **Oral presentation(Speed talk)**

  Effect of Kinetin in Polyphenol Accumulation and expression of Polyphenol oxidase gene (*PPO*) and Phenylalanine Ammonia-Lyase gene *(PAL*) in *Plantago ovata* Forsk *in vitro*-National Symposium on OXIDATIVE STRESS IN HEALTH AND DISEASE