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RESEARCH ARTICLE

-EFFECT OF DIFFERENT SEED PRIMING TREATMENTS ON ACTIVITY AND ISOZYME PATTERN OF ANTIOXIDANT ENZYMES IN OKRA

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Abstract

Effect of seed priming treatments and soaking durations on activity and isozyme pattern of antioxidant enzymes was studied. Four treatments:hydropriming, osmo-priming with 5% PEG, osmo-priming with 10% PEG and distilled water with soaking durations from 6 to 48 h at 6 h interval h were used. Dry okra seeds (unsoaked) seved as control. Priming increased the activity of antioxidant enzymes. Maximum increase was observed with T_2. Seeds soaked for 24 h showed maximum activity Priming with T_2 treatment for 24 h soaking gave the best results, followed by T_1 and T_3, while unsoaked control seeds proved to be the poorest.

Introduction

Okra (Abelmoschus esculentus L.) is one of the most widely known dicotyledonous plants and utilized species of the family Malvaceae (Naveed et al., 2009). It is a popular summer crop. The edible part of okra is the immature pod, which is harvested when tender. The young tender pods are cooked in curries, stewed and used in soups. Young okra leaves are also edible. Okra pods are a good source of flavonoid antioxidants like beta carotene, xanthein and lutein (Dilruba et al., 2009). Okra has been known to be beneficial to people suffering from leucorrhoea and general weakness. Due to its high iodine content, its fruits are considered useful to control goiter and have medicinal value in curing ulcers and relief from haemorrhoids (Demir 2001). This vegetable provides an important input of vitamins and mineral salts, including calcium which are often lacking in the diet of people in developing countries (Kahlon et al., 2007).

Seed priming is pre-sowing treatment used as a technique to enhance seed performance, notably with respect to rate and uniformity of germination (Taylor and Vananen, 1998), thereby improving seedling stand and enabling better crop establishment (Job et al 2000). It is a simple, low cost and effective approach for early seedling growth and yield under stressed and non stressed conditions. Seed soaking enhance the plant tolerance to salinity (Ehsanfer et al., 2006). Priming allows some of the metabolic processes to occur necessarily for germination before actual germination to get start (Yari et al., 2010). Priming triggers the synthesis or activation of some enzymes that catalyze the mobilization of storage reserves in seed, while endosperm weakens by hydrolase activities. Priming

may increase resistance to abiotic stresses (Farooq et al 2008). Primed seeds have been reported to give rise to crops, which matured earlier, and gave higher yields (Ndunguru and Rajabu, 2004). Priming could enhance the activity of antioxidative systems, resulting in lower rate of lipid peroxidation, contributing to seed invigoration and allows some of the metabolic process for germination to occur (Bradford, 1990). Priming increases the production of antioxidant enzymes like catalase, peroxidase, superoxide dismutase etc that help in protecting the cell against membrane damage because of lipid peroxidation (Varier et al., 2010).

To evaluate the best soaking sources and priming period for okra the present study was carried out to study the effect of seed priming treatment on germination and activity of antioxidant enzymes of okra. The objective of this study was to increase activity of antioxidant enzymes with seed priming treatments and soaking durations at very wide range of favoured and unfavoured environmental conditions.

MATERIALS AND METHODS

EXPERIMENTAL DETAILS AND SEED PRIMING

Experiment was conducted in laboratory of the department of Vegetable Science, Punjab Agricultural University, Ludhiana to evaluate the effect of seed priming treatments and soaking durations on activity of antioxidant enzymes and isozyme banding pattern of these enzymes of okra (Abelmoschus esculentus L.) viz. Punjab 8. Seed priming treatments included T_1 (hydro-priming), T_2 (osmo-priming with 5% Polyethylene glycol), T_3 (osmo-priming with 10% Polyethylene glycol) and T_4 (priming with distilled water). Seeds were fully immersed in priming solutions for soaking durations of 6, 12, 18, 24, 30, 36, 42 and 48 hours. Dry seeds were considered as control treatment that was 0 h soaked. To avoid fungal growth during the priming process, a fungicide Captan (2g/L) was added to the priming solutions. At the end of each priming period, the seeds were air dried at room temperature for at least 3 h close to original moisture level.

ENZYME EXTRACTIONS AND ASSAY

Catalase (Chance and Maehley, 1955)

Extraction

Sample (0.5g) was homogenized in 5ml of cold (4^{o}C) 0.1M Sodium phosphate buffer pH 7.0 containing 1% (w/v) insoluble PVP and 1mM EDTA. The extract was centrifuged at 20,000 rpm for 10 minutes at 4°C, the clear supernatant being taken for enzyme assay.

Assay

Catalase was assayed at 37°C in a reaction mixture containing 1.9ml of 0.1M sodium phosphate buffer pH 7.0 in 0.1ml of enzyme extract. The reaction was initiated by adding 1ml of H_2O_2 to the reaction mixture. The rate of decrease in absorbance at 240 nm was measured at 10 second intervals for 1 min. The specific activity of CAT was expressed in terms of unit of H_2O_2 decomposed per min per mg of protein.

Peroxidase (Shannon et al., 1966)

Extraction

Sample (0.2g) was homogenized in 2ml of cold (4^{o}C) 0.1M Tris-HCl buffer containing 1% (w/v) insoluble PVP, 1mM EDTA and 10µM β-mercaptoethanol. The extract was centrifuged at 20,000 rpm for 10 minutes at 4°C, the clear supernatant being taken for enzyme assay.

Assay

Reaction mixture contained 3ml of chilled guaiacol solution (111 µl of guaiacol was dissolved in 0.1M sodium phosphate buffer pH 6.5) and 0.1ml enzyme extract. The reaction was initiated by adding 0.1ml of H_2O_2 to the reaction mixture. The rate of decrease in absorbance at 470 nm was measured at 30 second intervals for 3 min. The specific activity of POX was expressed in terms of unit of H_2O_2 decomposed per min per mg of protein.

Esterase (Sae et al., 1971)

Extraction

Sample (0.2g) was homogenized in 5ml of cold (4^{o}C) 0.1M Sodium acetate buffer pH 7.0. The extract was centrifuged at 20,000 rpm for 10 minutes at 4°C, the clear supernatant being taken for enzyme assay.

Assay

Reaction mixture contained 4.6ml of 0.05M Phosphate buffer pH 8.0 and 0.2ml enzyme extract. The reaction was initiated by adding 0.2ml of 1mM indophenyl acetate to the reaction mixture. Esterase activity was assayed by change in absorbance at 625nm of 1.0/min. The specific activity of Esterase was expressed in terms of unit of indophenol produced per min per mg of protein.

Protein from Enzyme Extract (Lowry et al 1951)

Assay

To 0.2ml of appropriately diluted enzyme extract (to make total volume 1ml) was added 5ml of alkaline copper tartarate. Mixed well and the contents were allowed to stand for 10 minutes at room temperature. Then 0.5ml of Folin's reagent was added rapidly, mixed and contents were allowed to stand for 30 minutes. Intensity of blue color so developed was read at 520nm. Amount of protein in sample was calculated from standard curve prepared

simultaneously with bovine serum albumin (25-250µg/ml) as standard.

Malondialdehyde Content (Ohkawa et al 1979)

Extraction

Tissue (1g) was homogenized in 2.5ml of ice cold 0.1% TCA and centrifuged at 10000 x g at 4ºC for 15 minutes. Supernatant after centrifugation was used for estimation of malondialdehyde.

Assay

For estimation, 1ml of supernatant was mixed with 4ml of solution containing 0.5% thiobarbaturic acid and 20% TCA. Blank was prepared by taking 1ml of 0.1% TCA in place of supernatant. Test tubes were kept at 100ºC for 30 minutes and then cooled down to room temperature. Contents were centrifuged at 10,000 x g for 10 minutes. O.D. was read at 532 nm and 600 nm against blank. Absorbance at 600 nm was subtracted from absorbance at 532 nm. The MDA content was calculated by using molar extinction coefficient of malondialdehyde of 155 mM^{-1} cm. Results were expressed as nmole of MDA g^{-1} of fresh weight tissue.

ELECTROPHORETICAL SEPARATION OF ISOENZYMES (NATIVE-PAGE) (Schagger et al., 1994)

Enzyme extracts of catalase, peroxidise and esterase were analysed electrophoretically under non-denaturating conditions using Native-polyacrylamide. The freshly prepared separating gel solution was poured into the chamber without generating bubbles. Acrylamide solution was over-layered with H_2O saturated n-Butanol without mixing. Gel was polymerized for 1 hour. N-butanol was poured off from the polymerized separating gel; gel washed from top with water and remaining gap was filled in the chamber with stacking gel solution, after which comb was inserted. When the stacking gel polymerized; the comb was removed gently without distorting the shapes of the well. The tank of apparatus was filled with reservoir buffer Electrophoresis buffer pH 8.3 (28.8g 0.192M glycine, 6.0g 0.025M Tris, 2 lt distilled Water). Dissolved the sample extract in equal volume of 2x sample buffer. Equal amounts of enzyme dissolved in equal volume of 2x sample buffer (2.5ml 4x Stacking gel buffer, 2ml 20% glycerol, 40µl of 5% bromophenol blue, 5.5ml distilled Water) and equal amount were loaded onto each lane. A constant voltage of 200 V was applied for 5 h and the temperature maintained at 4 °C.

Gel Formation

Component	Separating gel	Stacking gel
30% Acrylamide solution	1.25 ml	0.4 ml
4x Buffer	2.5 ml (pH 8.8)	1.0 ml (pH 6.8)
Distilled water	6.25 ml	2.6 ml
APS	50 µl	20 µl
TEMED	10 µl	5µl

Enzyme activity staining

For visualisation of isozyme pattern gel was stained in the staining solution 250 ml (0.25g Coomassie Brilliant blue, 125ml Methanol, 25ml Glacial acetic acid and 100ml Distilled Water) for 4 hours. The unbound dye was removed in destaining solution 1 litre **(**100ml Methanol, 100ml Glacial acetic acid and 800ml Distilled Water) and fixed in 30% (v/v) ethanol.

STATISTICAL METHODS

Experimental units were arranged factorialy in a completely randomized design (CRD) with three replications. Mean ± S. D. was calculated and data was analyzed CRD at $p < 0.05$.

RESULTS AND DISCUSSION

Activity of Catalase Enzyme

Catalase activity was significant increase by various priming sources and soaking durations (Table 1) caused in CAT activity. Maximum CAT activity was recorded in T_2 followed by T_1 and T_3. Minimum CAT activity was recorded in T_4. Results of various soaking durations indicate that maximum increase in activity was recorded in seeds soaked for 24 h followed by 30 h, 36 h and 42 h, while minimum increase in activity was recorded in seeds soaked for 6 h. However, in their interaction, highest activity was recorded in seeds soaked for 24 h in T_2 followed by 30 h in T_3 and 24 h in T_3. Similar results reported by Del Ryo et al (2002) indicate that seed priming improves the antioxidant enzymes activity which decreases the adverse effects of reactive oxygen species. Similar results reported by Ella et al 2011 that the improved emergence and enhanced growth of rice, wheat and maize seedlings from primed seeds under environmental stress is caused by higher CAT and POX activities in primed seeds.

Activity of Peroxidase Enzyme

Seed priming treatments and soaking durations had significant effect on POX activity (Table 2). Maximum POX activity was recorded in T_2 followed by T_3 and T_1 as compared to control. POX activity was significantly affected by soaking durations. Maximum activity was recorded in the seeds soaked for 24 h duration followed 30 h, 36 h and 42

h, while minimum activity was recorded in 12 h soaking duration. Interaction effects due to seed priming treatment and soaking durations showed difference in POX activity. The highest activity was recorded in seeds soaked for 24 h in T_2 followed by T_1. The lowest activity was recorded in seeds soaked for 6 hours in T_1. Similar results were shown by Wahid et al (2008) that antioxidant activity of POX improved with priming, under salt stress in maize, rice, wheat and sunflower. Increased peroxidase activities are reported under abiotic stresses (Gill and Tuteja 2010).

Activity of Esterase Enzyme

Esterase is a hydrolyzing enzyme that splits ester into acid and an alcohol in a chemical reaction with water called hydrolysis. Increased esterase activity has been reported in various types of stress affecting plants and it has been suggested as a defense mechanism against oxidative damage (Lambert et al 1999 Andres et al 2001). The data on the esterase activity was found to be statistically at par in okra and has been reported in Table 3. With respect to esterase activity, significant differences were noticed among seed priming treatments and soaking durations. Maximum esterase activity was recorded in T_2 followed by T_1. Various soaking durations indicate that the seeds soaked for 24 h showed maximum activity followed by 36 h and 42 h, while minimum activity was recorded in 6 h soaking duration. However, in their interaction, highest activity was recorded in seeds soaked for 24 h in T_1 followed by 36 h in T_2 and 30 h in T_3. The lowest activity was recorded in seeds soaked for 6 hours in T_3. Similar results were shown by Jie et al (2002) that activities of antioxidant enzymes (esterase, catalase) were found to be higher in mungbean seeds after Osmopriming as compared to those in control.

Malonidialdehyde content (MDA)

The data on the MDA content was found to be statistically at par in okra and has been reported in Table 4. Various priming sources and soaking durations cause significant decrease in MDA content in okra seed. Maximum decrease in MDA content was recorded in seeds soaked in T_2 followed by T_1 and T_3 as compared to control. Results of various soaking durations indicate that maximum decrease in MDA content was recorded in seeds soaked for 24 h followed by 30 h, 42 h and 36 h, while minimum decrease in MDA content was recorded in 6 h soaking duration. It has been reported that the content of soluble sugars in the germinated seedlings after osmopriming enhanced, leading to higher tolerance to osmotic stress (Yuan-Yuan et al 2010). Priming also decreased MDA and increased CAT and POX activities in dry seeds before sowing (Jubany-Mari et al 2010). The positive effect on reducing the extent of lipid peroxidation was also observed in lucerne seedlings (Zhang et al 2007).

Table 1: Catalase activity in okra seedlings during germination in response to various soaking durations and seed priming treatments

SOAKING DURATIONS (h)	(TREATMENTS)					
	CONTROL	**T_1**	**T_2**	**T_3**	**T_4**	**Mean**
0	20.02±1.18 (1.682±0.021)	-	-	-	-	**20.02**
6	-	23.84±0.74 (2.961±0.028)	20.34±1.21 (2.695±0.026)	20.69±1.25 (2.733±0.022)	21.49±0.62 (2.792±0.025)	**20.84**
12	-	21.58±1.25 (2.734±0.019)	23.98±1.07 (2.988±0.029)	20.36±1.39 (2.685±0.024)	21.96±2.28 (2.832±0.031)	**21.97**
18	-	24.13±2.66 (3.182±0.032)	24.79±2.31 (3.074±0.021)	24.93±1.93 (3.084±0.029)	23.68±1.46 (2.985±0.025)	**24.38**

24	-	28.68±1.78 (3.931±0.031)	34.68±1.44 (3.896±0.034)	30.96±2.38 (3.591±0.035)	24.59±1.36 (2.053±0.039)	**29.73**
30	-	26.92±1.39 (3.436±0.026)	30.19±1.42 (3.526±0.029)	32.44±2.20 (3.732±0.033)	23.24±1.40 (2.944±0.021)	**28.20**
36	-	31.59±1.33 (3.655±0.033)	27.21±2.26 (3.279±0.022)	28.40±2.13 (3.374±0.024)	24.06±1.68 (3.049±0.024)	**27.82**
42	-	26.04±1.29 (3.171±0.031)	28.44±1.45 (3.381±0.026)	26.39±2.09 (3.196±0.035)	21.89±1.46 (2.843±0.037)	**25.69**
48	-	30.71±1.34 (23.592±0.022)	26.89±2.13 (3.256±0.034)	24.75±1.49 (3.061±0.025)	20.90±2.04 (2.762±0.020)	**24.18**
Mean	**20.02**	**26.69**	**27.07**	**26.18**	**22.73**	**25.35**
CD 5%	A=Seed priming treatments=0.536 B = Soaking durations=0.758 A*B=1.516					

Table 2: Peroxidase activity in okra seedlings during germination in response to various soaking durations and seed priming treatments

SOAKING DURATIONS (h)	(TREATMENTS)					
	CONTROL	T_1	T_2	T_3	T_4	Mean
0	2.66±0.30 (0.179±0.012)	-	-	-	-	**2.66**
6	-	2.62±0.32 (0.276±0.019)	3.94±0.33 (0.363±0.025)	3.03±0.36 (0.300±0.015)	2.74±0.25 (0.283±0.025)	**3.67**
12	-	2.80±0.29 (0.283±0.018)	3.38±0.23 (0.322±0.024)	3.33±0.08 (0.322±0.022)	2.56±0.15 (0.270±0.017)	**3.09**
18	-	2.69±0.23 (0.278±0.014)	4.97±0.18 (0.434±0.021)	3.12±0.13 (0.301±0.026)	3.13±0.18 (0.304±0.012)	**3.48**
24	-	5.95±0.12 (0.491±0.025)	7.73±0.20 (0.612±0.020)	4.87±0.17 (0.424±0.023)	3.28±0.20 (0.316±0.016)	**5.46**
30	-	4.28±0.15 (0.382±0.017)	5.18±0.25 (0.442±0.024)	4.62±0.26 (0.402±0.021)	2.79±0.36 (0.286±0.023)	**4.43**
36	-	3.72±0.17 (0.344±0.021)	5.54±0.36 (0.361±0.018)	4.78±0.30 (0.387±0.014)	3.08±0.15 (0.308±0.028)	**4.28**
42	-	6.03±0.10 (0.506±0.019)	4.11±0.34 (0.374±0.010)	4.25±0.26 (0.345±0.024)	2.96±0.20 (0.294±0.019)	**3.77**
48	-	3.70±0.28 (0.241±0.023)	3.14±0.29 (0.203±0.017)	3.72±0.16 (0.249±0.027)	2.83±0.38 (0.288±0.020)	**3.35**
Mean	**2.66**	**3.60**	**5.00**	**3.72**	**3.26**	**3.89**
CD 5%	A=Seed priming treatments=0.247 B = Soaking durations = 0.350 A*B = 0.700					

Peroxidase activity is expressed as units min^{-1} g^{-1} FW
Values in parenthesis indicate specific activity (µmoles per mg of the protein) of the enzyme

Table 3: Esterase activity in okra seedlings during germination in response to various soaking durations and seed priming treatments

SOAKING DURATIONS (h)	(TREATMENTS)					
	CONTROL	T_1	T_2	T_3	T_4	Mean
0	2.51±0.17 (0.314±	-	-	-	-	**2.51**
6	-	2.68±0.23 (0.335±0.018)	3.42±0.19 (0.472±0.021)	2.12±0.27 (0.269±0.014)	2.89±0.25 (0.366±0.012)	**2.78**
12	-	3.19±0.21 (0.389±0.023)	4.80±0.23 (0.613±0.025)	3.01±0.25 (0.367±0.020)	2.51±0.30 (0.319±0.017)	**3.38**
18	-	4.81±0.24 (0.610±0.026)	4.68±0.30 (0.558±0.024)	3.92±0.27 (0.493±0.023)	3.23±0.19 (0.413±0.022)	**4.16**
24	-	7.93±0.15 (1.13±0.029)	5.15±0.24 (0.858±0.027)	5.11±0.25 (0.634±0.026)	4.09±0.13 (0.516±0.019)	**5.69**
30	-	5.32±0.24	4.40±0.38	5.98±0.30	2.08±0.23	**4.45**

		(0.656±0.024)	(0.553±0.022)	(0.774±0.028)	(0.226±0.011)	
36	-	5.06±0.23 (0.623±0.025)	6.49±0.29 (0.812±0.026)	5.09±0.24 (0.623±0.027)	3.13±0.29 (0.382±0.016)	**4.94**
42	-	4.80±0.14 (0.609±0.021)	4.92±0.21 (0.651±0.018)	4.96±0.29 (0.612±0.019)	4.16±0.20 (0.527±0.018)	**4.71**
48	-	3.15±0.27 (0.339±0.016)	4.27±0.22 (0.534±0.015)	3.19±0.27 (0.387±0.015)	2.21±0.21 (0.279±0.013)	**3.20**
Mean	**2.51**	**4.62**	**4.77**	**4.17**	**3.10**	**4.16**
CD 5%	A=Seed priming treatments=0.268 B = Soaking durations=0.379 A*B=0.759					

Esterase activity is expressed as units min^{-1} g^{-1} FW
Values in parenthesis indicate specific activity (µmoles per mg of the protein) of the enzyme
T_1 = Hydropriming
T_2 = Osmo-priming with 5% PEG
T_3 = Osmo-priming with 10% PEG
T_4 = Priming with distilled water

Table 4: Malondialdehyde content (MDA) in okra seedlings during germination in response to various soaking durations and seed priming treatments

SOAKING DURATIONS (h)	**TREATMENTS**					
	CONTROL	**T_1**	**T_2**	**T_3**	**T_4**	**Mean**
0	16.23±0.74	-	-	-	-	**16.23**
6	-	16.90±0.67	16.62±0.99	15.34±0.22	16.13±0.36	**16.25**
12	-	15.65±0.47	13.72±0.32	16.27±0.38	17.84±0.59	**15.87**
18	-	12.48±0.61	13.52±0.30	14.55±1.57	13.07±0.84	**13.41**
24	-	9.23±0.44	8.40±0.57	8.03±0.59	11.31±0.79	**9.24**
30	-	8.88±0.24	8.52±0.67	10.40±0.76	12.37±0.94	**10.04**
36	-	9.25±0.42	9.12±0.30	12.53±0.88	11.62±0.93	**10.63**
42	-	13.21±0.47	11.62±0.59	9.23±0.41	10.34±1.23	**10.40**
48	-	10.56±0.98	9.41±0.38	11.43±0.96	13.53±0.96	**11.23**
Mean	**16.23**	**12.07**	**11.37**	**12.22**	**13.28**	**12.22**
CD 5%	A=Seed priming treatments=0.355 B=Soaking durations=0.503 A*B = 1.006					

Malondialdehyde content is expressed µmoles g^{-1} of FW
Values are mean ± S.E. of three determinations

Isozyme pattern of antioxidant enzymes

Native-PAGE analysis of germinating okra in response to various seed priming treatments and soaking durations showed that the isozyme(s) bands of catalase, peroxidase and esterase (Fig 1, 2 and 3) were more intense in different seed priming treatments as compared to control. The increase in intensity of bands was also relatively higher in response to priming with T_2 as compared to that in T_1 and T_3, which show the higher activity of catalase enzyme in T_2 at 24 h soaking duration. Similar results were showed by Araby and Hagazi (2011) that progressive enhancements in the intensity of bands were recorded with different seed priming treatments. A new isozyme form of esterase was observed in T_2 treated seeds at 24 h duration. Increased activity of esterase has been reported in various type of stress affecting plants. The native-PAGE analysis of peroxidase showed higher number of bands as compared to catalase. A new isozyme form of peroxidase enzyme was observed in T_2 and T_1 treated seeds at 24 h duration. Enhanced peroxidase activity may play important role in the lignification of cell walls, which assists in the resistance to penetration by pathogens (Montes et al 2004). Corbineau et al (2000) have reported that, i.e higher number of bands of peroxidase isozyme, in response to osmo priming and hydro priming.

Fig 1 Isozymes banding pattern of catalase after seed priming treatments

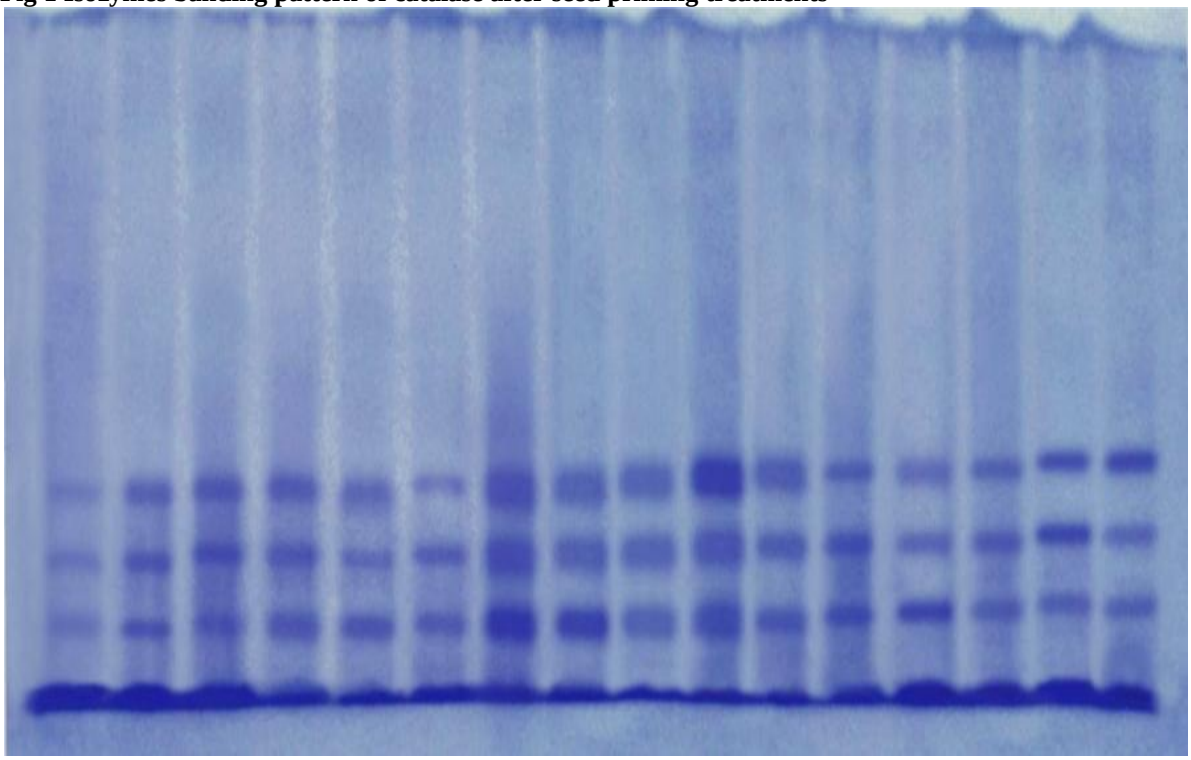
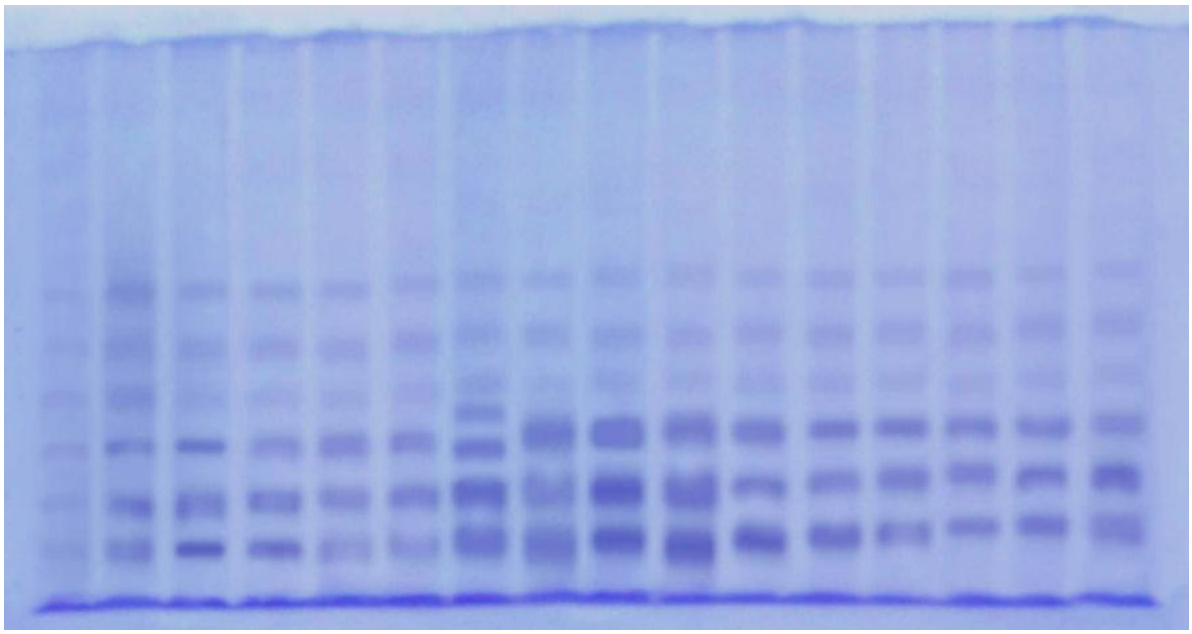
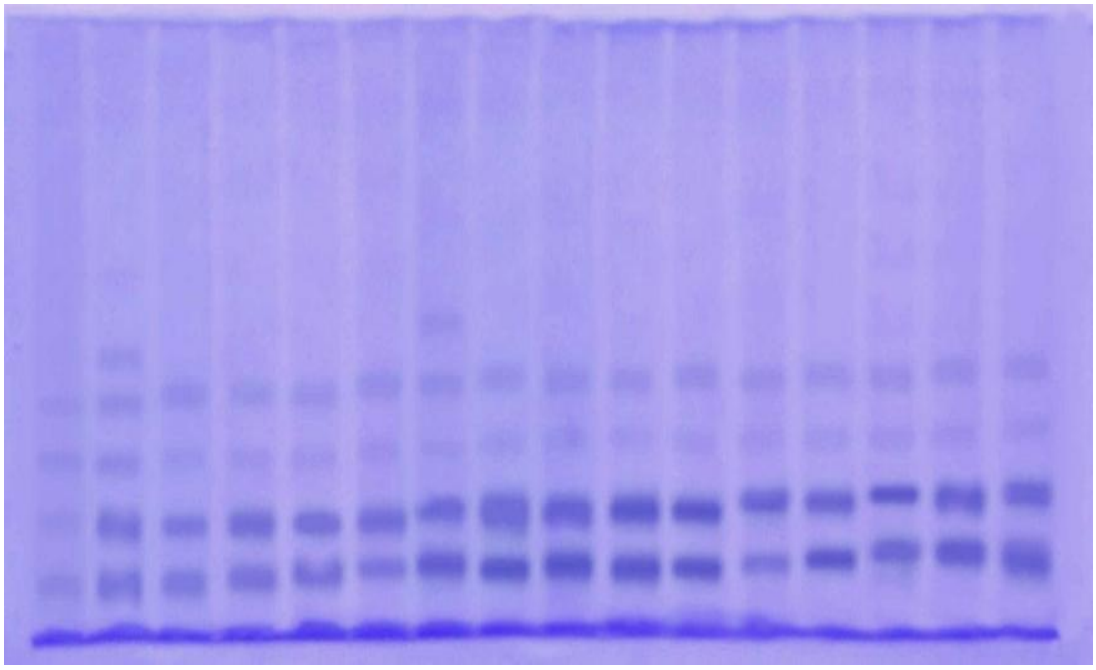


Fig 2 Isozymes banding pattern of esterase after seed priming treatment



C T_1 T_1 T_1 T_1 T_1 T_2 T_2 T_2 T_2 T_2 T_3 T_3 T_3 T_3 T_3
24h 30h 36h 42h 48h 24h 30h 36h 42h 48h 24h 30h 36h 42h 48h

Fig 3 Isozymes banding pattern of peroxidase after seed priming treatment



C T_1 T_1
T_1 T_1 T_1 T_2 T_2 T_2 T_2 T_2 T_3 T_3 T_3 T_3 T_3
24h 30h 36h 42h 48h 24h 30h 36h 42h 48h 24h 30h 36h 42h 48h

C - Control (untreated)
T_1 - Hydropriming
T_2 - Priming with 5% PEG (Polyethyleneglycol)
T_3 - Priming with 10% PEG (Polyethyleneglycol)
Soaking durations - 24 h, 30 h, 36 h, 42 h, 48 hours

CONCLUSIONS

It has been concluded from the research work that seed priming treatments resulted in increased activity of antioxidant enzymes than un-primed and distilled water primed seed. Seed priming with T_2 treatment may be used for enhancing emergence, better seedling growth and activity of antioxidant enzymes in okra, but both T_1 and T_3 treatments gave almost equal and better results. Okra seed priming with T_2 treatment (osmo-priming with 5% PEG) for 24 h duration lead to better germination and tolerate to adverse environmental effects by increasing activities of antioxidant enzymes of okra can be recommended to farmers.

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