PEROXIDASE ACTIVITY DURING IN VITRO GROWTH OF SALT STRESSED OCIMUM TENUIFLORUM. L

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ABSTRACT

The behavior of peroxidase activity of Ocimum tenuiflorum under NaCl stress during its in vitro growth was evaluated. The in vitro growth of O.tenuiflorum was achieved on MS basal medium supplemented with different PGRs and their different combinations at 24C° with 5.7 pH, 16 hrs photoperiod and 30g/l sucrose. Leaf explants gave the best callus growth i.e 90%, with a combination of NAA and Kinetin, (5.0+0.5mg\L), to which different salt concentrations of NaCl (0.1, 0.3, 0.4mg\l) were applied. Salinity significantly influenced the callus morphology of Ocimum tenuiflorum as greenish callus completely turned into black on the application of high salt concentrations. High concentration of salt strongly inhibited the plant growth and increased the peroxidase activity by activating the antioxidant enzyme system. After salt application peroxidase activity incearsed from, 0.992 to 1.913 n units / µg protein for the leaf callus during its 8th subculture. It was concluded during the present study that peroxidase activity levels can work as biochemical marker of tolerance of the plants towards biotic and abiotic stresses.

Key words: Ocimum tenuiflorum, PGRs, biochemical markers,Salt stress.

INTRODUCTION

Salinity is one of most threatening abiotic stresses to plants as saline area is three times greater than the agricultural land throughout the world, however many plants naturally adapt tolerance toward salt stress such as Halophytes, and xerophytes etc (Boscaiu *et al*., 2008).Salinity, significantly limits the plant growth as several physiological pathways photosynthesis, respiration, carbohydrate metabolism etc are affected by salt stress (Jani, 2010).

Ocimum tenuiflorum L.belongs to family Lamiaceae. Ocimum tenuiflorum is native of "Tropical Asia"and afterwards was distributed all over the world. The enhancement of tolerance against salt stress is proved to be an essential step towards progress in agricultural world. Plant tissue culture introduced an efficient method of organogensis and plantlet regeneration from callus. (Gogoi and kumaria 2011) reported that O.tenuiflorum developed frequent shoot formation when cultured on MS basal medium having 26.85µM, NAA and 02.32 µM Kinetin. This technique provide a key to control the stress homogeneity and cellular behavior (compartmentalisation of vacuoles) under salinity (Lutts *et al*.,2004).Plant tissue culture was proved to be better than conventional breeding programmes as it provided fundamental research tools to understand the adaptation of plants toward salt stress (Rain., 1980).

The present study was conducted to understand the behavior of peroxidase activity of O. tenuiflorum during its in vitro growth under the salt stress, in the controlled environment. The salt stress affects the enzyme functioning which in turn remodifies the plant's morphological and functional behaviors. Literature also indicate that under salt stress, The activity of antioxidant enzymes such as Ascorbate peroxidase and Glutathione reductase, increase (Hernandez *et al*, 2000).The intensity of salinity on Ocimum species has been observed by (Qadir *et al*., 2008) which showed that the concentration of salt increased the other aspects of growth such as plant height, fresh weight etc. decreased.

Present investigation also supports the idea that salt tolerance varies from in vivo to in vitro plants of O.tenuiflorum, not only depending upon its genome but also on physical and chemical factors present during its growth. Genetic analysis by (Zhu 2002) explained that salt tolerance is being induced by compartmentalization inside the plant cells and the vacuoles store the excessive amount of salt to prevent the salt damage to the plants.

(Sairam *et al*., 2002) reported that Plants adapted two types of mechanisms for scavenging "Reactive Oxygen Species"i:e enzymatic and non- enzymatic (α-tocophelrol ,ascorbic acid and caroteniods) reactions.

It is being reported by different workers that salt stress increased the catalase, ascorbate peroxidase, polyphenoloxidase, glutathione reductase and peroxidase activities in Fox-tail mellet, Canola and Soyabean showing the changes in their Morphological characters i:e root and shoot length, fresh and dry weight etc (Sreenivasulu *et al*., 1998, Hernandez *et al*., 2000, Shan, 2006: Dolatabadian *et al*.,2009 and Welsany *et al*., 2012). Hence plant peroxidase provides conventional tools as

biochemical markers for understanding plant responses towards both biotic and abiotic stress. (Ashraf and Harris, 2004).

Data of present study suggested that peroxidase activity increased along with an increase in the applied salt concentration, while the morphological appearance and characters were deregulated (most probably by a negative effect on physiological processes like respiration, photosynthesis, carbohydrate metabolism etc. It was also noted during the present work peroxidase activity estimation can work as an indicator of salt stress, tolerated by the plant.

MATERIALS AND METHOD

Experimental procedure was divided into following two steps:

- In vivo growth of Ocimum tenuiflorum including aseptic plantlets from seeds
- In vitro growth of Ocimum tenuiflorum.

Leaf explants used for this study were collected from ornamental plants cultivated in Lahore College for Women University, Lahore. The Tulsi explants collected from the field were washed several times under running tap water with commercial detergent for 15-20 minutes without damaging the young and the delicate tissue. Sterilized seeds were grown on cotton beds covered with filter paper in petri plates.

Fig No.1. In vivo seed germination

Fig No. 2. In vitro seed germination

MS medium (Murashige and Skoog, 1962) was used throughout the study supplemented with different growth hormones. MS medium stock solutions were prepared by mixing the inorganic and organic components using general scheme of (Tasaki 1986).It was sterilized by autoclaving under pressure of 15 Psi for 15 minutes at 121˚C and then stored at 4˚C till its use. All the cultures were maintained at 24±1ºC, under photoperiod of 16 hours light, provided by white fluorescent tubes with the intensity of 2000-3000 lux at relative humidity 75-80%.The explants were inoculated on media with single and different combinations of plant growth regulators as NAA, 2, 4-D, Kn. and BAP. The callus ‘s morphological characters were recorded. Interval for sub culturing was 3 weeks..Different concentrations of NaCl were used in the present investigation and added into already prepared MS medium. MS medium was supplemented with 3 different salt concentrations (0.1, 0.3, 0.4 mg\L) to detect the effect of salt stress on Ocimum tenuiflorum callus cultures. Each treatment was repliated 15 times and the experiment was repeated thrice. The 15 days NaCl treated callus cultures were then shifted to regeneration medium. The experiment was planned with 30 culture vessels per NaCl treatment and it was repeated three times. At day 30, regeneration frequency, number of shoots per culture vessel and mean shoot length were recorded. The regenerated shoots were shifted to MS basal medium for further shoot development for 30 days and then were transferred to the already standardized rooting medium for each cultivar. Data for root formation percentage, and root length number of roots per culture vessel were recorded at day 30, after shifting the regenerated plants (60-days-old) to the rooting medium. For quantitative analysis of peroxidase, the method proposed by (Racusen and Foote 1965) was employed to estimate the amount by spectrophotometer. Optical density was measured at 470nm. The optical density increased linearly for 40 seconds. The specific activity of enzyme was measured at the increase of O.D at 470nm/mg/40seeconds. . The results obtained were statistically analyzed. The means

were separated by Duncan's new multiple range test at 1% level of significance as described by (Steel., *et al* 1997).

RESULTS

As mentioned above, the present investigations composed of two main steps.

a: In vitro growth including callus induction, maintenance and plant regeneration

b: Evaluation of peroxidase activity of O.tenuiflorum growth under salt stress

a. In vitro growth including callus induction, maintenance and plant regeneration.

Different growth regulators and combination of different growth regulators were used for callogenesis from leaf explant etc. Callus initiated after 12 days after inoculation while a mature callus was under observation after 60 days.

Table 1: Effect of Constant concentration of NAA (5.0mg\L) with different concentrations of Kin on in vitro growth of O tenuiflorum

Combination of Growth Regulators	Callus Intiation %age	Callus growth	Callus colour	Callus texture	LSD VALUE
NAA+Kin					
5.0+0.5	90±0.56[a]	+++	Dark Green	Compact buded	1.29
5.0+2.5	70±0.30[b]	+++	Green	Compact	
5.0+5.0	30±.61[c]	+	Yellowish green	Fariable	
NAA+ BAP					
5.0+0.5	80±.21[ab]	+++	Blackish green	Compact	1.54
5.0+2.5	30±.58[b]	+	Green	Loose	
5.0+5.0	20±0.54[ab]	+	Yellow	Friable	

Fig 3: Callogenesis from leaf explant of Ocimum tenuiflorum turning black after 15 days of inocculation in MS (Murashige & Skoog, 1962) medium supplemented with NAA and Kinetin 5.0 mg/l 2,4-D+ 0.5mg/l 0.3 ml NaCl.

b.

After 60 days of callus formation three concentrations of NaCl (0.1,0.3,0.4mg\L) were applied to the MS medium. The salt concentrations turned callus from green to black. The intensity of black color increased with an increase of salt concentration. At day 10th of subculture, callus turned into brownish green at 0.1mg\L NaCl as shown in table 2

Table 2: Morphological characters of Ocimum tenuiflorum callogenesis with application of different concentrations of NaCl concentrations in MS medium supplemented with NAA $ Kin 5.0+0.5.

Sr No.	Salt Concentrations (mg\L)	Morphological callus characters before Salt (Na Cl) application	Morphological callus characters after Salt (Na Cl) application		
		At 60 day	Day 10	Day20	Day30
1	0	Dark Green color ,compact	Dark Green color ,compact	Dark Green color ,compact	Dark Green color, compact
2	0.1	Dark Green color ,compact	Brownish green, compact	Blackish green Compact	Black compact
3	0.3	Dark Green color ,compact	Brownish green	Blackish green Compact	Black compact
4	0.4	Dark Green color ,compact	Blackish green compact	Black ,compact	Black, compact

b: Evaluation of peroxidase activity of O.tenuiflorum growth under salt stress

Peroxidase activity in salt stressed calli from leaf explants is mentioned in table 3. The result has depicted that leaf calli at subculture 8th showed the maximum peroxidase activity (0.919±0.0057) before addition of NaCl. However after salt application peroxidase activity incearse (1.913 ± 0.01153). The percentage of peroxidase activity increased with an increased in salt concentration.

Fig. 4: Callogenesis from leaf explant of Ocimum tenuiflorum turning black after 15 days of inocculation in MS (Murashige and Skoog, 1962) medium supplemented with NAA and Kinetin (5.0 mg/l 0.5mg/l) 0.3 ml NaCl

Table: 3. Specific activity of peroxidase (n units / µg protein) subcultures calli of leaf explants of O.tenuiflorum on MS medium supplemented with NAA (5.0mg/L):

No of Culture	Specific activity of Peroxidase (n units / µg protein)	
	Leaf explant callus before salt (NaCl) application	Leaf explant callus after salt (NaCl) application
1st Subculture	0.599 ± 0.0057^{g}	1.193 ± 0.0057^{bc}
2nd Subculture	0.621 ± 0.0112^{f}	1.343 ± 0.0100^{ab}
3rd Subculture	0.651 ± 0.0063^{e}	1.512 ± 0.0230^{b}
4th Subculture	0.701 ± 0.0057^{d}	1.717 ± 0.0068^{a}
5th Subculture	0.719 ± 0.0010^{c}	1.995 ± 0.0047^{a}
6th Subculture	0.823 ± 0.0580^{b}	1.613 ± 0.493^{ab}
7th Subculture	0.919 ± 0.0046^{a}	1.322 ± 0.0100^{c}
8th Subculture	0.992 ± 0.0050^{a}	1.913 ± 0.01153^{cd}

± = Standard error

Means within columns followed by different letters were significantly different at P =0.05 according to Duncan's multiple range test.

DISCUSSION

Ocimum tenuiflorum is one of the important medicinal plants. To reduce the pressure on naturally occuring resources, it is important to produce different plant cultivars, using modern technologies. Plant tissue culture is one of the very effective techniques for this purpose. During present investigation best response towards callogenesis was observed by leaf explants and friable callus developed on MS basal medium supplemented with 5.0+0.5mg/l NAA+Kin.. (Shilpa, 2010) suggested the same media for callogenesis by using the same explant. However the media they used was supplemented with different PGR's i.e 2mg/l NAA and 0.2mg/l Kin.

(Lukman-ul- Hakim., *et al* 2007) reported best callus formation on same medium but supplied with different conc of PGR (0.1- 0.5mg/l) with an interval of 0.1 mg/l NAA.+1.0mg/l Kinetin (Jaggi *et al* (2003) reported that from internode of Ocimum tenuiflorum, good callus formation was resulted in the conc. of 2.0mg/l 2, 4-D in MS basal medium

Peroxidase activity was maximum (0.919±0.0057) at 8th subculture before addition of salt. However after salt application peroxidase activity increased (1.913 ± 0.01153). The percentage of peroxidase activity increase with an increase in salt concentration increases.

The ability of plants to evolve mechanisms to detoxify toxic chemicals produced inside the cytoplasm allowed them to successfully grow in adverse environmental conditions (Weigel *et al*., 1990). It has also been suggested that an important consequence of salt stress is the generation of reactive oxygen species (Mittler, 2002). It was further reported earlier that salinity affects important metabolic processes located in chloroplasts and mitochondria (Cheeseman, 1988) but little is known about its effect on activated oxygen metabolism of these organelles. Plants are reported to produce high amount of enzymatic and non-enzymatic antioxidants, i.e., superoxide dismutase, catalase, peroxidase, ascorbic acid and glutathione (Neto *et al*., 2006).

It was concluded during the present study that peroxidase activity estimation can work as a bioindicator for salt stress tolerance of a plant. Hence plant peroxidase profiles can be used as biochemical markers for understanding plant responses towards both biotic and abiotic stresses. The present investigation provides a base for the further research and study of salt stress effects on enzymatic activity of a plant. By understanding the enzymatic mechanism during salt stress, one may be able to understand the relevant pathways, helpful for inducing tolerance against salt and other stresses by regulating the specific enzymatic actions.

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