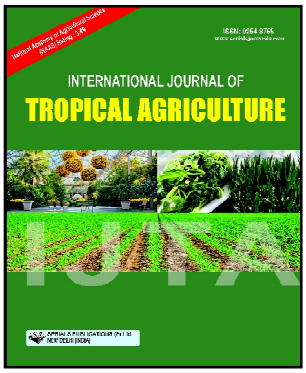

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Phenyl propanoid pathway leads to drought stress tolerance in castor (*Ricinus communis* L.)

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Abstract: Drought stress is one of the major abiotic stresses in agriculture worldwide. Present study was carried out to investigate the effect of drought stress on phenol profiling, proline content, total protein, polyphenol oxidaes, enzymes of phenyl propanoid pathway (Phenylalanine ammonia lyase, p-Coumaric acid 3-hydroxylase and Cinnamic acid 4- hydroxylase) and expression of genes of phenylpropanoid pathway (*PAL, C4H1and C4H2*) in drought tolerant castor (*Ricinus communis* L.) genotype 48-1. A pot experiment was carried out in a complete block design with three replications. Water deficit was induced by polyethylene glycol (PEG) that leads to biochemical and molecular changes in castor. Treatments included control (no drought), -0.2 MPa, -0.3 MPa, -0.4 MPa and -0.5 MPa water potential. Treatment was given at seedling stage and all the parameters were studied in leaves and roots of castor at 0, 24, 48 and 72 hour after treatment (hat). Based on the present investigations it was found that caffeic acid and ferulic acid are important phenolics that were detected in leaves of castor, while gallic acid was additionally detected in roots of castor. Thus, these three phenolics may serve as important biochemical marker for large scale screening of drought tolerant genotypes in castor. Activity of PPO and phenylpropanoid pathway (PAL, C4H and C3H) enzymes were studied at different time points with different levels of drought stress in castor leaves and roots. Expression analysis of key genes of phenylpropanoid pathway (*PAL*, *C4H1* and *C4H2*) revealed maximum fold expression of *PAL* and *C4H2* in the leaves, whereas minimum fold expression was observed for *C4H1*. Thus, it may be concluded that phenylpropanoid pathway is an important biochemical pathway that leads to drought stress tolerance in castor.

Key words: *Ricinus communis* L., drought stress, phenylpropanoid pathway, phenolics, HPTLC, RT-PCR

INTRODUCTION

Castor (*Ricinus communis* L.) is an industrially important non edible oil seed crop. Castor oil and its derivatives are used in the manufacturing of soaps, lubricants, hydraulic and brake fluids, paints, dyes, coatings, inks, cold resistant plastics, waxes and polishes, nylon, pharmaceuticals and perfumes (Mutlu and Meier, 2010). Clinically, castor oil is used to stimulate labor (Anon., 2007). Drought stress is one of the main causes for crop yield reduction in the majority of agricultural regions of the world. Drought stress induces several physiological, biochemical and molecular responses in several crop plants, which would help them to adapt to such limiting environmental conditions (Bajaj *et al.,* 1999; Arora *et al.,* 2002). It inhibits the photosynthesis of plants, causes changes of chlorophyll contents and damage to the photosynthetic apparatus (Escuredo *et al.,* 1998). It also inhibits the photochemical activities and decreases the activities of enzymes in the Calvin cycle (Monakhova and Chernyadev, 2002). The alteration of antioxidant metabolisms is one of the fundamental metabolic processes that may influence the drought tolerance of perennial grasses (Da Costa and Huang, 2007). A common effect of drought stress is the disturbance between the generation and quenching of reactive oxygen species (ROS) (Smirnoff, 1998). Reactive oxygen species (ROS) are the byproducts of many degenerative reactions in crop plants, which will affect the regular metabolism by damaging the cellular components (Foyer and Noctor, 2002). A ubiquitous feature of plant response to drought stress is apparently the enhanced activation of phenylpropanoid pathway, suggesting general defensive role for phenylpropanoid compounds. It is hypothesized that the molecular basis for the protective action of phenylpropanoids in plants is their antioxidant and free radical scavenging properties (Korkina, 2007). Phenylpropanoid metabolism triggers a cascade of biochemical reactions which lead to the production of important phenolic compounds involved in defense responses of a host plant. Changes in host steady state mRNA levels during a drought stress can provide a valuable readout of the molecular processes (Sestili *et al.,* 2011). The complex resistance response provoked in plant during drought stresses have been studied and characterized at the molecular level to a large extent in the model plant *Arabidopsis thaliana* and to a lesser extent in agronomically important crops. So, an experiment was conducted in castor to investigate the effect of water stress on phenolic content, activity of enzymes and expression of genes of the phenylpropanoid pathway.

MATERIALS AND METHODS

The investigation was carried out using drought tolerant castor genotype 48-1, which was procured from castor and pulse research station, NAU, Navsari.

Seed sowing: Castor seeds were raised in plastic cup under the controlled condition at Department of Plant Molecular Biology & Biotechnology, ASPEE College of Horticulture and Forestry, NAU, Navsari. Sand used for seed sowing was washed several times with deionised water to leach out the salts. Further the sand was autoclaved and used for seed sowing. Seeds were sown on the surface of the sand and then covered with a 2 cm layer of sand. Four plants in each glass were kept up to 20 days after germination. The plants were kept in greenhouse to maintain appropriate temperature & humidity. Plants were well irrigated with half-strength Hoagland solution regularly to avoid any physiological stress and to maintain high relative humidity condition.

Pot transplanting: Castor seedlings (20 days old) were planted in 14 cm × 12 cm pots using sterile and thoroughly washed sand. Plants were grown for three weeks at 25/10 °C day/night temperatures in a greenhouse and watered on need basis with modified half strength Hoagland nutrient solution.

Treatment: Three week old seedlings of castor were treated with PEG-6000 to create artificial

drought stress levels of 0.0 (control), -0.2, - 0.3, -0.4, and -0.5 MPa by dissolving 11.9, 15.1, 17.8 and 20.2 g of PEG-6000 per 100 ml distilled water, respectively as described by Radhamani *et al.* (2012). Leaves and roots were pulled out and washed with distilled water and about 1-2 cm roots were cut from terminal sides after 0, 24, 48 and 72 hour. Samples without stress were treated as control.

Biochemical analysis: All biochemical parameters were analyzed at 0, 24, 48 and 72 hat in the leaves & roots of seedlings. Fresh second upper leaf and root samples were collected at each stage for analysis and washed twice with tap water followed by deionised water. Leaves & roots without mid-rib were analyzed in three repetitions for following biochemical estimation.

Phenol profiling: (a) Extraction of phenolics - Plant phenolics were extracted and separated as the method described by Mahatma *et al.,* (2011). One gram of leaf and root tissues were ground in liquid N_2 to a fine powder and phenolics were extracted with 80% methanol at 70 °C. Homogenates were centrifuged at 5000 rpm for 10 min. The extraction procedure was repeated four times on the pellet. The supernatants were pooled and made up to 25 ml with 80% methanol. The supernatants were evaporated to dryness under water bath at 70 °C. The residue obtained was dissolved in 1 ml of HPLC grade methanol and depigmented with activated charcoal. (b) Standard phenolics - Six phenolic compounds (coumaric, ferulic, gallic, caffeic, salicylic and catechol) were obtained from Hi-Media and Sigma. Catechol was dissolved in deionised water and rest of the phenolics were dissolved in HPLC grade methanol (10 mg ml^{-1}). From these standards, 100 ìl of each standard were taken and volume made up to 1 ml with HPLC grade methanol to get a final conc. of 1 mg ml^{-1}. (c) HPTLC analysis of phenolics - Extracted samples (6 ìl) were sprayed as 1x6 mm bands along with standards (6 ìl) on HPTLC aluminium plate 20x10 cm (Silica gel 60 F254, Merck) by CAMAG Linomat 5 applicator using nitrogen gas. The plates were activated at 100 °C for 20 min before sample application. For separation of phenolics the solvent system was prepared and derivatized as method described by Sadasivam and Manickam (2008). Phenolics were separated by the solvent system chloroform:acetic acid (9:1). Solvent mixture was filled in the CAMAG twin through chamber (20x10 cm) and left overnight for saturation. The plates were placed in tank to separate phenolics until solvent reached 8 cm from origin. Qualitative and Quantitative analysis - After separation of phenols, HPTLC plates were photographed using CAMAG Reprostar 3 system at 254 nm under UV light. Quantitative determination was carried out using CAMAG TLC Scanner 3 at 254 nm.

Enzyme activity of polyphenol oxidase (PPO; EC 1.14.18.1): Enzyme extraction – Castor leaf and root tissues (300 mg) were ground in 3 ml of 0.2 M potassium phosphate buffer, pH 6.8. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was used for enzyme assay. Enzyme assay - The reaction mixture contained 2.9 ml of catechol (0.05 M catechol in 0.2 M phosphate buffer pH 6.8) and reaction was initiated by the addition of 0.1 ml of enzyme extract. The changes in the colour due to the oxidized catechol were read at 490 nm for three minutes at an interval of 30 second. The enzyme activity was expressed as change in O.D. min^{-1} g^{-1} protein (Thimmaiah, 2006).

Enzyme activity related to phenylpropanoid pathway: (a) Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) - Enzyme extraction: Leaf and root tissues (300 mg) were homogenized with a pre chilled mortar and pestle in 3 ml of extraction buffer containing 50 mM borate- HCl buffer (pH 8.5) and 0.04% â-mercaptoethanol. The homogenate was centrifuged at 10,000 rpm for 15 min. The clear supernatant was used as the enzyme source for the assay of PAL. Enzyme assay: The reaction mixture contained 3.0 ml of 0.1 M sodium borate buffer (pH 8.8) and 0.5

ml of 0.1 M phenylalanine (dissolved in 0.1 M sodium borate buffer, pH 8.8). The reaction was initiated by the addition of 0.1 ml enzyme extract. The tubes were incubated at 37 °C for 2 h. The blank was also set with substrate. The O.D. was read at 290 nm after 2 hr. The enzyme activity was expressed as ìM cinnamic acid hr^{-1} g^{-1} protein. (Mahatma *et al.,* 2011). (b) Cinnamic acid-4-hydroxylase (C4H; EC 1.14.13.11) - Enzyme extraction: Leaf and root tissues (100 mg) was ground in a chilled mortar and pestle with 5 ml 100 mM Tris-Cl buffer (pH 7.5) and extract was centrifuged at 2000 rpm for 20 min at 0 °C. The supernatant was used for C4H assay. Enzyme assay: The reaction mixture contained 2 ml of Tris-Cl buffer (pH 7.5), 0.25 ml each of β-mercaptoethanol, 8 mM cinnamic acid, 8 mM NADPH and 0.5ml of enzyme extract. The reaction was stopped by adding 0.1 ml 6 M HCl after incubating at 30°C for 1 h and extracted twice with 5 ml of diethyl ether. The organic phase was evaporated to dryness and residue was dissolved in 3 ml of 1 M NaOH. p-Coumaric acid production was measured by spectrophotometer at 340 nm. Standard curve was prepared by dissolving p-coumaric acid in 1 M NaOH. The enzyme activity was expressed as µM p-coumaric acid hr^{-1} g^{-1} protein (Padhiar and Sharma, 2008). (c) p-Coumaric acid-3-hydroxylase (C3H; EC 1.14.14.1) - Enzyme extraction: Enzyme extraction method was similar as described for C4H. Enzyme assay: The assay mixture contained the same constituents as for C4H assay, except that cinnamic acid was replaced by p-coumaric acid. The reaction was allowed to proceed for 30 min at 30 °C and was terminated by addition of 6 M HCl. The reaction mixture was extracted with diethyl ether, after evaporating the organic phase, the residue was dissolved in 0.5 ml of absolute methanol and caffeic acid production was measured by spectrophotometer at 316 nm. Standard curve was prepared by dissolving caffeic acid in absolute methanol. The enzyme activity was expressed as µM caffeic acid hr^{-1} g^{-1} protein (Padhiar and Sharma, 2008).

Molecular analysis: Expression analysis of key genes of phenylpropanoid pathway was carried out at four stages: 0, 24, 48 and 72 hat with different concentrations of drought stress in both treated and non treated plants. (a) RNA extraction - Fresh leaf and root tissues (100 mg) were collected at 0, 24, 48 and 72 hat from treated and non treated control plants and powdered in liquid nitrogen using autoclaved mortar and pestle. The resulting powder was transferred to a 1.5 ml tight capped eppendorf tube and RNA was extracted using TRIzol (Invitrogen) reagent. The integrity of RNA was assessed by denaturing agarose gel electrophoresis and quantification was carried out by nano spectrophotometer. The RNA samples with the ratio of 1.7–2.0 at OD 260/280 were retained. RNA was separated by denaturing agarose gel electrophoresis. The 0.8% denaturing gel was prepared in 1.0x formaldehyde agarose gel buffer (Sambrook and Russell, 2001). Gel was run in 1x formaldehyde agarose gel running buffer at 5–6 V/cm till the dye reaches three-fourth of gel. When the 28S and18S RNA resolved by electrophoresis, exhibited at near 2:1 ratio on ethidium bromide staining indicated that no significant degradation of RNA occurred. (b) cDNA synthesis - The cDNA was synthesized by using high capacity cDNA reverse transcription kit (Fermentas) following manufacturer's instructions. For cDNA synthesis, 2 µl of template RNA (100 ng), 1µl of Oligo(dT) primer (0.5 µg) and 8 µl of nuclease free water were mixed by brief centrifugation and incubated at 65 °C for 5 min followed by immediate cooling on ice. After that 4 ìl of 5x reaction buffer, 1 µl of RibolockRNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix and 2 ìl of M-MuLV Reverse Transcriptase (20 U/µl) were mixed by brief centrifugation and incubated for 60 min at 37 °C. The reaction was terminated by heating at 70 °C for 5 min. The reverse transcription reaction products (cDNA) were stored at e20 °C until used. Quantitative RT-PCR analysis of *PAL* and *C4H* genes - The amplification and expression analysis

of *PAL, C4H1 and C4H2* genes were performed using specific primers. The gene sequences of *PAL, C4H1* and *C4H2* were searched from castorDB, http://castordb.msubiotech.ac.in (Thakur *et al.,* 2011) and primers were designed using primer 3 software. Sequences of primers used in present study are mentioned in Table 1. RT-PCR was performed using a 1 µl aliquot (100 ng/reaction) of the first strand cDNA in a final volume of 10 µl containing 10 pmol of specific primers (forward and reverse) for coding region of *PAL* and *C4H* genes. As a control, the primer specific to ubiquitin gene was used as to normalize each sample for variations in the amounts of RNA used. PCR was carried out using 5 µl 2x QuantiFast SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), Primer F and R both were 0.5 µl, Template cDNA 1 µl and RNase free water 3 µl to final volume 10 µl in a thermal cycler (ABI-7300). Real time PCR program was run as an initial denaturation for 5 min at 95 °C, 35 amplification cycles at 95 °C for 10 s (denaturation) and 30 s annealing at 60 °C, 64 °C and 68 °C for *PAL, C4H-1 and C4H-2* primers, respectively. Each sample was tested in triplicate for all primers. Melting curve analysis was performed on all samples to ensure amplification of a single product with the expected melting temperature and the absence of primer-dimers. The products of each primer set were tested by agarose gel electrophoresis to verify that a single product of the expected size was produced. Relative RNA quantities were determined with the delta-delta (ΔΔ)Ct, according to the following formula (Dussault and Pouliot, 2006) comparing the data for each gene of interest with the data for mock-inoculated control samples at each time point. The data were normalized by comparison to reference (castor ubiquitin) gene. ΔΔ Ct = [(Ct G.O.I Ctr e Ct Ref. Ctr) e (G.O.I treated e Ct Ref. treated) where: G.O.I. = gene of interest; Ref = reference gene (ubiquitin), Ctr = control (non-treated) and Fold increase = $2^{-\Delta\Delta Ct}$.

RESULTS AND DISCUSSION

Biochemical analysis

Phenol profiling - Total three phenolic acids were observed during drought stress in castor. Caffeic acid and ferulic acid were observed in drought treated leaves and roots at different water potentials and at different time intervals (Fig 1: A-H). The caffeic acid content of leaves is presented in Fig. 2. Caffeic acid content of roots is shown in Fig. 4. Caffeic acid content increased in drought treated leaves and roots up to 72 h. While, the percent area of caffeic acid was highest in drought treated leaves at -0.5 MPa water potentials at 72 hat. and the percent area of caffeic acid was also highest in drought treated roots at same water potentials and same time points. The relative percentage of caffeic acid increased by

Table 1
Sequences of primers used for quantitative RT-PCR analysis

Sr. No.	*Primer*	*Sequence (5'-3')*	*NCBI accession no.*
1	PAL F	CAAGTTGCTGCTATTGCTAGCC	30038.m002319
	PAL R	TCCATGGATTCACCATTAGGGC	
2	C4H1 F	GCAGTTCCGGCCAGAGCGAT	43540.m000048
	C4H1 R	GCAGCTCCGCCTGCCCATAC	
3	C4H2 F	CTCCCTCCGGGACCTATGCC	29976.m000504
	C4H2 R	ACACGCGCCGCCTCATCTTC	
4	RcUBIQ F	TCTTCTTAGGCCTTAACTGATTGC	XM 002530294.1
	RcUBIQ R	ATGGCTATGGCTGGATTGTACC	

129.93% at -0.5 MPa water potentials at 72 hat compared to control in castor leaf, whereas the relative percentage of caffeic acid increased only by 47.74% with the same water potentials and same time points compared to control in castor root. Ferulic acid was also observed in drought treated leaves at 0, 24, 48 and 72 hat (Fig. 1: A-H). The ferulic acid content of leaves is presented in Fig. 3. Ferulic acid content of roots is shown in Fig. 5. Ferulic acid content increased in drought treated leaves and roots up to 72 hat. The percent area of ferulic acid was highest in drought treated leaves and roots at -0.5 MPa water potential at 72 hat. The relative percentage of ferulic acid increased by 66.06% with -0.5 MPa water potential at 72 hat compared to control in castor leaf, whereas in roots there was an increase of 60.69% with the same water potential and same time points compared to control. Gallic acid was observed only in drought treated roots at 24, 48 and 72 hat but not observed in root as control and drought treated leaves (Fig. 1: A-H). The gallic acid content of roots is presented in Fig. 6. The percent area of gallic acid was detected higher in the treated roots at -0.4 MPa water potential at 24 hat.

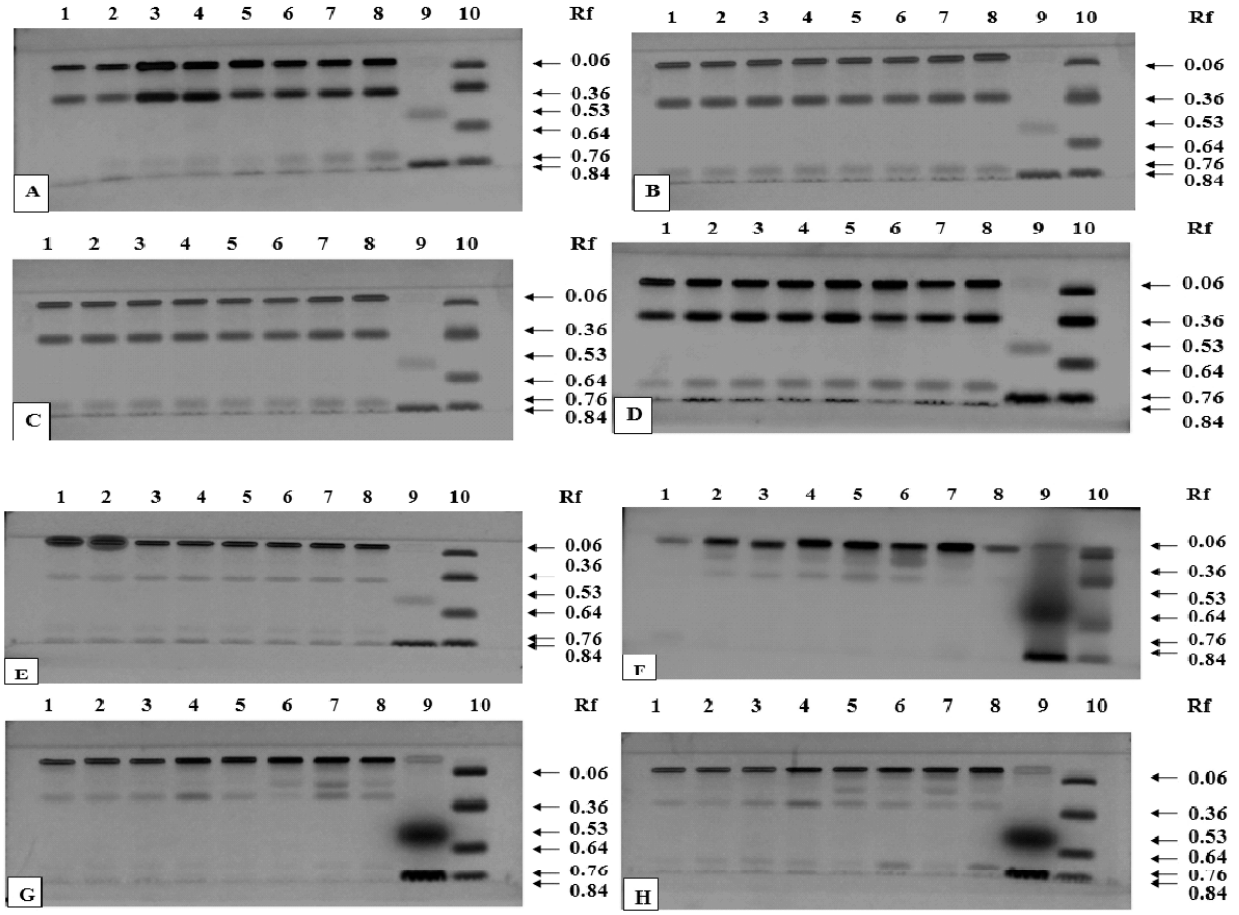

Figure 1: Phenol profiling from castor genotype 48-1 leaves and roots during drought stress. (A) Leaf sample at 0 hat (B) Leaf sample at 24 hat (C) Leaf sample at 48 hat (D) Leaf sample at 72 hat (E) Root sample at 0 hat (F) Root sample at 24 hat (G) Root sample at 48 hat (H) Root sample at 72 hat. [1 - control, 2 – Treatment with 50 mM NaCl, 3 - Treatment with 100 mM NaCl, 4 - Treatment with 200 mM NaCl, 5 - Treatment with -0.2 MPa water potential, 6 - Treatment with -0.3 MPa water potential, 7 - Treatment with -0.4 MPa water potential, 8 - Treatment with -0.5 MPa water potential, 9 - Mixture of standards: catechol (Rf: 0.53) and salicylic acid (Rf: 0.84), 10 - mixture of standards: p-coumaric acid (Rf: 0.64) ferulic acid (Rf: 0.76), gallic acid (Rf: 0.06) and caffeic acid (Rf: 0.36)].

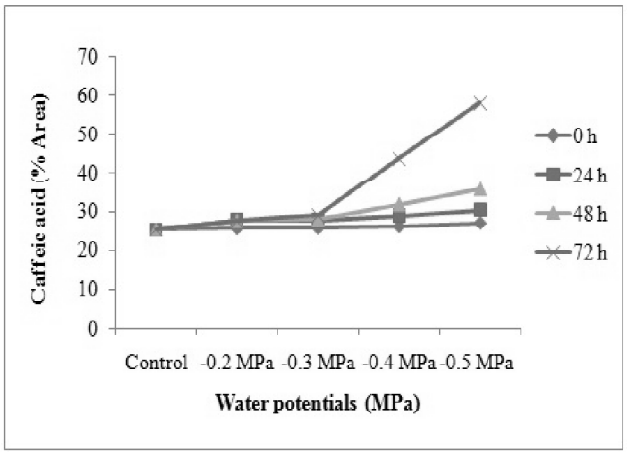

Figure 2: Caffeic acid in leaves at 0, 24, 48 & 72 hat during drought stress

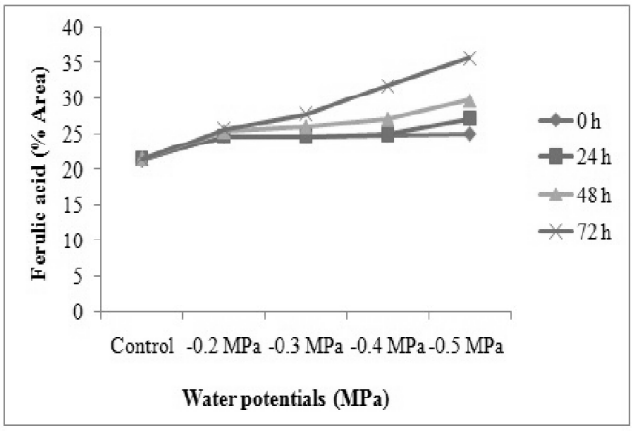

Figure 3: Ferulic acid in leaves at 0, 24, 48 & 72 hat during drought stress

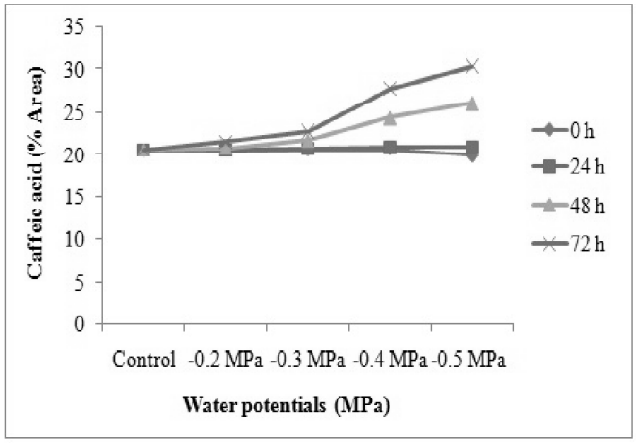

Figure 4: Caffeic acid in roots at 0, 24, 48 & 72 hat during drought stress

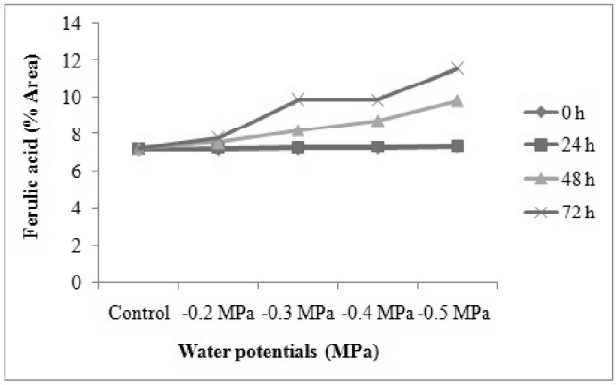

Figure 5: Ferulic acid in roots at 0, 24, 48 & 72 hat during drought stress

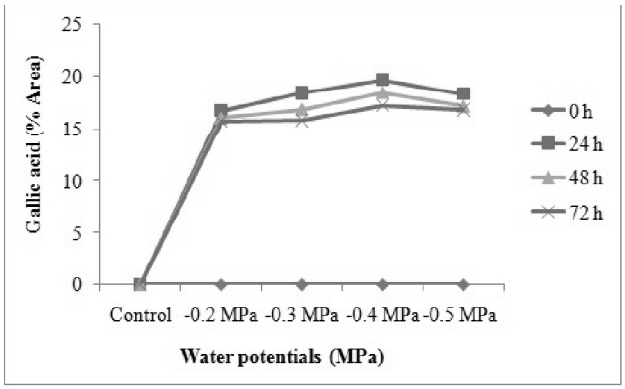

Figure 6: Gallic acid in roots at 0, 24, 48 & 72 hat during drought stress

In the present study caffeic acid and ferulic acid are important phenolics observed in leaves and roots of castor and their level is higher after treatment with PEG as compared to non treated control. Similarly, an increase of caffeic acids was observed by Alvarez *et al.,* (2008) in the xylem sap of maize. This might be due to the high radical scavenging activity of caffeic acid under drought stress (Grace and Logan, 2005). In contrast a reduction in the amount of ferulic acid in the xylem sap of maize was also observed by Alvarez *et al.* (2008). In the present study the enzyme responsible for production of ferulic acid is C3H that is found in an increasing order up to 72 hat which may be correlated with the ferulic acid production. Gallic acid was observed only in drought treated roots and not observed in drought treated leaves. Saheb *et al.,* (2010) observed that all PEG (5%, 10%, 15% and 100%) treatments

significantly increased the total soluble phenols contents under oxidative stress condition. Results observed in this study are in accordance with Goodman *et al.,* (1967), who found multi fold increase of phenols af-ter challenging with elicitation might be due to the excess production of H_2O_2 in elicited plant cells through increased respiration or due to the activation of hexose-monophosphate pathway, acetate pathway and release of bound phenols by hydro-lytic enzymes. An increase in total phenolic content as a response to drought stress was reported in numerous reports (Abreu and Mazzafera, 2005). Polyphenols act against stress in plants, however, dependent on the stress factor different phenol groups or single compounds are synthesized and accumulated (Dixon and Paivia, 1995).

Enzyme activity of polyphenol oxidase (PPO): The PPO enzyme activity was measured in leaves and roots of castor at different water potentials and at different time points. PPO enzyme activity in leaves is presented in Fig. 7, whereas PPO enzyme activity in roots is given in Fig. 8. PPO enzyme activity in leaves and roots of castor increased up to 48 hat during drought stress. The highest PPO enzyme activity was observed in leaves at 48 hat with -0.5 MPa water potentials that showed increase of 1.37 fold compared to control. In root PPO enzyme activity was found highest at the same time point and same water potentials that was 1.24 fold higher than control.

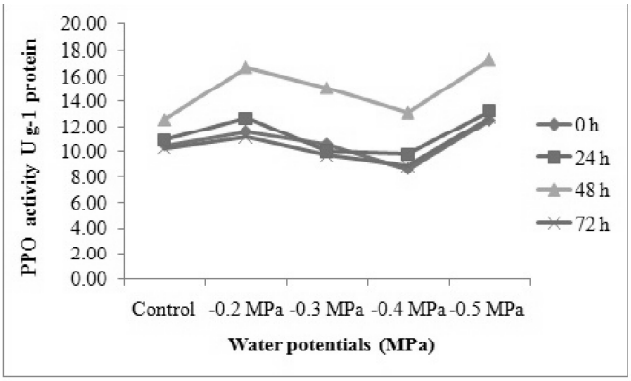

Figure 7: PPO activity in leaves at 0, 24, 48 & 72 hat during drought stress

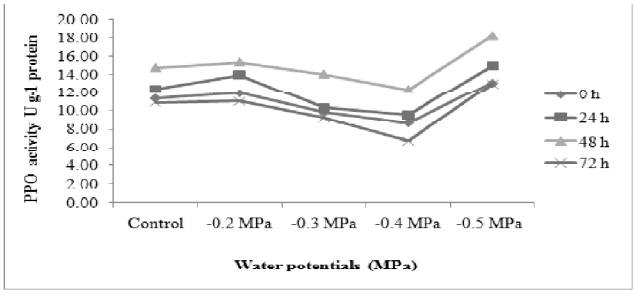

Figure 8: PPO activity in roots at 0, 24, 48 & 72 hat during drought stress

Enzyme activity related to phenylpropanoid pathway: (a) Activity of phenylalanine ammonia lyase (PAL): PAL activity increased during drought stress. PAL activity in the leaves is presented in Fig. 9, whereas in root PAL activity is shown in Fig 14. PAL activity increased up to 72 hat. The highest PAL activity was observed in leaves at 72 hat with -0.2 MPa water potential that was 1.87 fold higher compared to control. In root PAL activity was highest at 72 hat with -0.4 MPa water potential that was 1.27 fold higher than control.

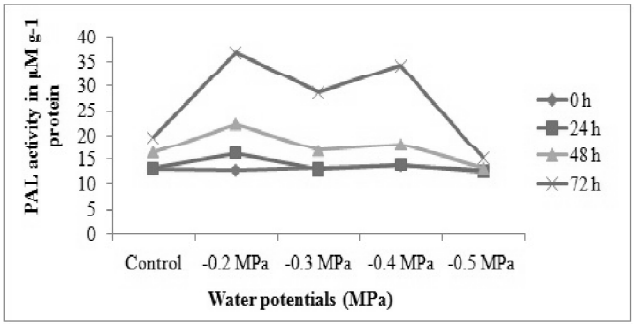

Figure 9: PAL activity in leaves at 0, 24, 48 & 72 hat during drought stress

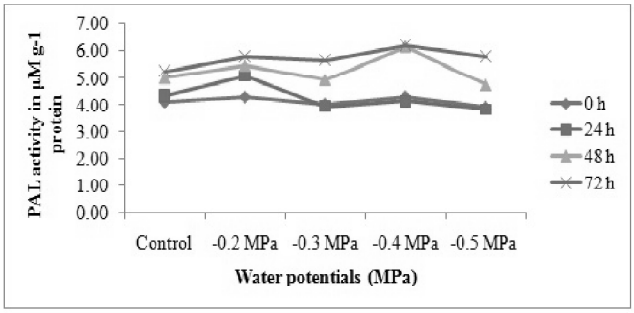

Figure 10: PAL activity in roots at 0, 24, 48 & 72 hat during drought stress

(b) Activity of cinnamate 4 hydroxylase (C4H): The C4H enzyme activity was measured in leaves and roots of castor at different water potentials and at different time points. The C4H enzyme activity of leaves and roots are presented in Fig. 11 and Fig. 12 respectively. The C4H enzyme activity increased up to 72 hat. Maximum C4H enzyme activity in the leaves was observed at 72 hat with-0.4 MPa water potential that is 1.34 fold increase compared to untreated control. Similarly maximum C4H enzyme activity in the roots were observed at the same time with -0.5 MPa water potential that is 1.26 fold increase compared to untreated control. From these results it was observed that C4H enzyme activity is higher in leaves than roots during drought stress.

(c) Activity of p-Coumaric acid 3-hydroxylase (C3H): The C3H enzyme activity was measured in leaves and roots of castor at different water potentials and at different time points. The C3H enzyme activity of leaves and roots are presented in Fig. 13 and Fig. 14 respectively. The C3H enzyme activity increased up to 72 hat. Maximum C3H enzyme activity in the leaves was observed at 72 hat with -0.4 MPa water potential that is 1.19 fold increase compared to untreated control. Maximum C3H enzyme activity in the roots were observed at the same time with -0.3 MPa water potential that is 1.83 fold increase compared to untreated control. From these results it may be concluded that C3H enzyme activity is higher in roots than leaves during drought stress.

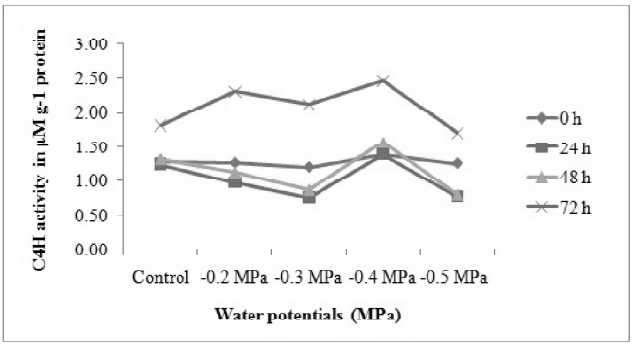

Figure 11: C4H activity in leaves at 0, 24, 48 & 72 hat during drought stress

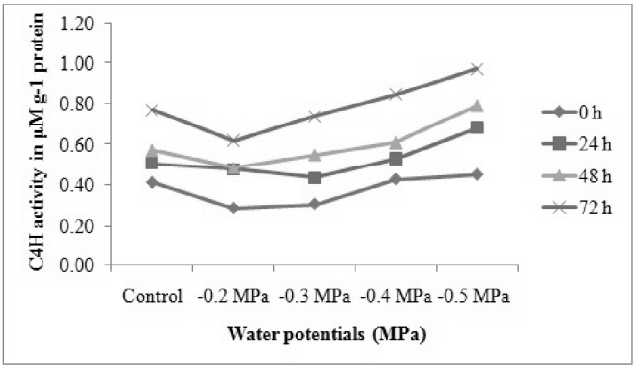

Figure 12: C4H activity in roots at 0, 24, 48 & 72 h during drought stress

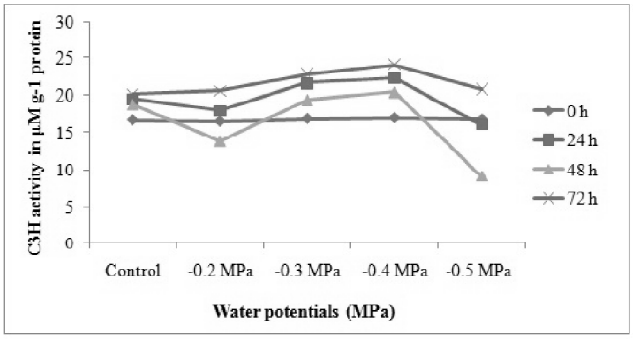

Figure 13: C3H activity in leaves at 0, 24, 48 & 72 hat during drought stress

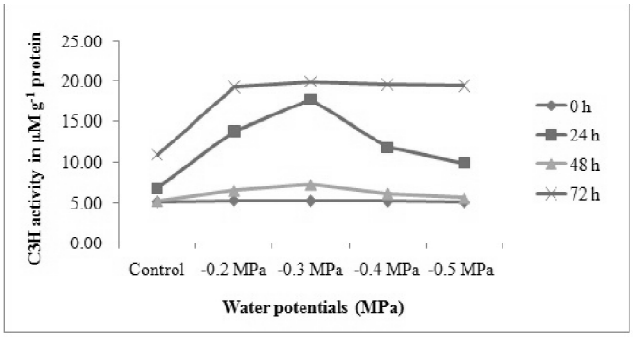

Figure 14: C3H activity in roots at 0, 24, 48 & 72 hat during drought stress

PPO enzyme activity was highest at 48 hat in leaves and roots during drought stress. Similar result was also reported by Zivkovic *et al.,* (2010) in *Asplenium ceterach* tissue during dehydration treatments compared to the control. Vaziri *et al.,* (2011) also reported that the average activity of PPO in leaves of *A. Lagopoides* increased under weak withholding water stress, whereas under medium and strong stresses it decreased over control. Moreover,

this value in *A. lagopoides* was significantly higher to *A. litoralis*. Thus, higher levels in *A. lagopoides* compared to *A. littoralis* showed that this species was more tolerant to the stress. The activity of PAL significantly correlates with increasing drought stress in leaves as well as roots of castor. Similarly, an increase in the activity of PAL enzyme was recently reported in the cases of *winter triticale* and a drought resistant maize genotype (Hura *et al*., 2008; Hura *et al*., 2007). The sharp peaks of PAL during one day stress period in the leaves of both maize inbreeds was also observed by Gholizadeh, (2011) and in leaves of rice cells (Saheb *et al.,* 2010). Guidi *et al.* (2008) also proved that activity of PAL enzyme increased by 16% in response to water stress and the activity of PAL enzyme was linearly correlated with phenolic compounds. In this study PAL enzyme activity in roots increased up to 72 hat but no significant changes were observed in drought treated roots as compared to control. Similarly, no significant changes were observed by Gholizadeh, (2011) for PAL enzyme activity in the root tissues of both maize inbreeds during stress period as compared to control and they found that in roots, the activity of PAL enzyme remained constant. So, it seems that PAL enzyme is a responsible antioxidative enzyme during drought stress in the leaves, but not in the roots. As like PAL, the enzyme activity of C4H was maximum at 72 hat in leaves as well as roots of castor during drought stress. Similar result was reported by Li-ping *et al.* (2009) *When Prunus mongolia* Maxim leaves were subjected to drought stress, they observed the enzyme activity of PAL and C4H increased gradually when drought stress increased that shows positive correlation between PAL and C4H enzyme activity and plant drought resistance. Reduced C4H enzyme activity has also been correlated with reduced levels of Klason lignin (Blount *et al.,* 2000). In the present investigation also a positive correlation has been observed in the drought stress and C4H enzyme activity. The enzyme activity of C3H was found maximum at 72 hat during drought stress. Similar results were obtained with *Citrullus lanatus* sp., which shows an extraordinary resistance to drought (Yoshimura *et al*., 2008). It was also observed that there was an increased expression of genes involved in lignin biosynthesis during the intermediate and final stages of water stress (from 48 to 72 h), such as those coding for PAL, C3H, 4-coumarate: coenzyme A ligase (4CL), caffeoyl coenzyme A *O*-methyltransferase (CCoAOMT), cinnamyl alcohol dehydrogenase (CAD) and peroxidase.

Molecular analysis

Expression analysis of PAL gene using Real Time PCR: Expression of *PAL* gene was studied in leaves and roots of castor at different water potentials and at different time points. *PAL* gene expression of leaves and roots are presented in Fig. 15 and Fig. 16 respectively. Maximum *PAL* gene expression in the leaves was observed at 72 hat with -0.4 MPa water potential that increased by 38.62 fold as compared to control (Fig. 15). Similarly, maximum *PAL* gene expression in the roots was observed at the same time point and same water potential that increased by 18.64 fold as compared to control (Fig. 16). At lower water potential *PAL* gene expression was found higher in leaves and roots but at severe water potential *PAL* expression decreased.

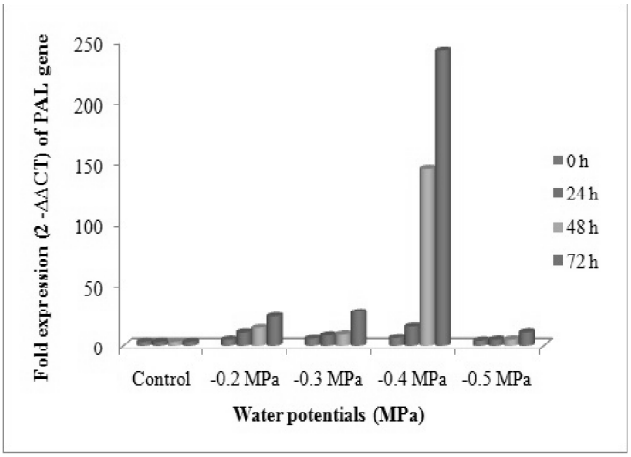

Figure 15: Comparative real time PCR results in fold expression ($2^{-\Delta\Delta CT}$) of *PAL* gene in leaves of castor during drought stress

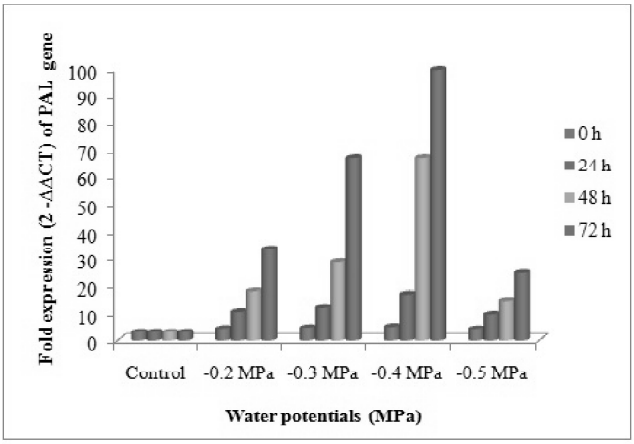

Figure 16: Comparative real time PCR results in fold expression ($2^{-\Delta\Delta CT}$) of *PAL* gene in roots of castor during drought stress

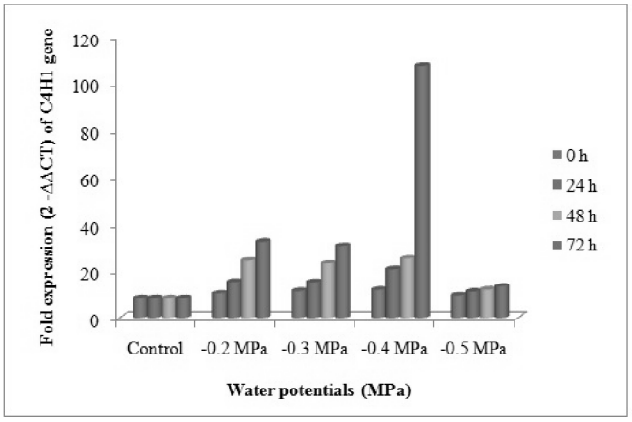

Figure 18: Comparative real time PCR results in fold expression ($2^{-\Delta\Delta CT}$) of *C4H1* gene in roots of castor during drought stress

Expression analysis of C4H 1 gene using Real Time PCR: Expression of *C4H1* gene was studied in leaves and roots of castor at different water potentials and at different time points. *C4H1* gene expression of leaves is presented in Fig. 17, whereas *C4H1* gene expression of roots are given in Fig. 18. *C4H1* gene expression in leaves and roots increased up to 72 hat. Maximum *C4H1* gene expression in the leaves was observed at 72 hat with -0.4 MPa water potential that is increased by 9.98 fold as compared to control (Fig. 17). Similarly, maximum *C4H1* gene expression in the roots was observed at the same time point and -0.5 MPa water potential that increased by 9.06 fold as compared to control (Fig. 18). In leaves gene expression was found higher as compared to roots during drought stress.

Expression analysis of C4H2 gene using Real Time PCR: Expression of *C4H2* gene was studied in leaves and roots of castor at different water potentials and at different time points. *C4H2* gene expression of leaves and roots are presented in Fig. 19 and Fig. 20 respectively. Maximum *C4H2* gene expression in the leaves was observed at 72 hat with -0.5 MPa water potential that increased by 55.69 fold as compared to control (Fig. 19). Similarly, maximum *C4H2* gene expression in the roots was observed at the same time point with same water potential that increased by 32.22 fold as compared to control (Fig. 20). In

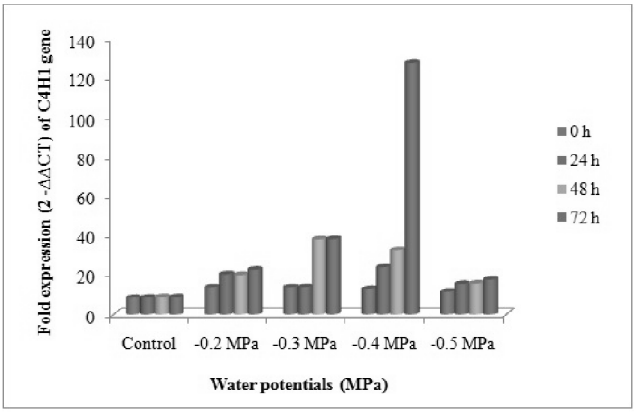

Figure 17: Comparative real time PCR results in fold expression ($2^{-\Delta\Delta CT}$) of *C4H1* gene in leaves of castor during drought stress

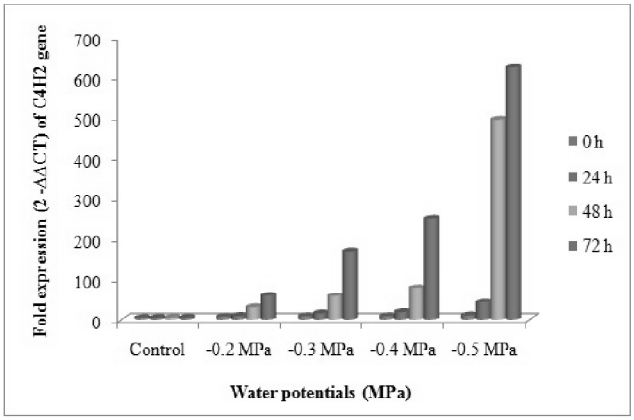

Figure 19: Comparative real time PCR results in fold expression ($2^{-\Delta\Delta CT}$) of *C4H2* gene in leaves of castor during drought stress

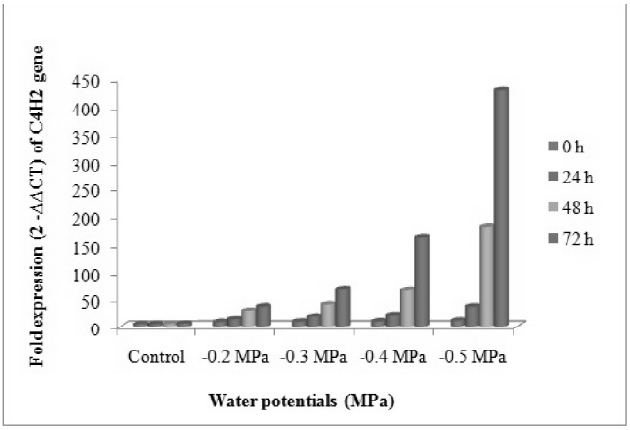

Figure 20: Comparative real time PCR results in fold expression ($2^{-\Delta\Delta CT}$) of *C4H2* gene in roots of castor during drought stress

leaves gene expression was found higher as compared to roots during drought stress.

Expression analysis of key genes of phenylpropanoid pathway (*PAL*, *C4H1* and *C4H2*) revealed maximum fold expression of *PAL* and *C4H2* in the leaves, whereas minimum fold expression was observed for *C4H1*. Very few studies have been carried out on expression of *PAL* gene from roots under abiotic stress condition however, in a similar study Pawlak-Sprada *et al.* (2011) observed species-specific changes in expression of phenylalanine ammonia-lyase (*PAL*) and lignin content in roots of soybean (*Glycine max* L.) and lupine (*Lupinus luteus* L.) seedlings treated with different concentrations of cadmium (Cd^{2+}, 0–25 mg l^{-1}) or lead (Pb^{2+}, 0–350 mg l^{-1}). The expression of *PAL* was enhanced in both plant species at higher metal concentrations (15–25 mg l^{-1} of Cd^{2+}or 150–350 mg l^{-1} of Pb^{2+}); however it was not directly correlated with *PAL* mRNA. This suggests a transcriptional and posttranscriptional control of *PAL* expression under heavy metals stress. In soybean, Cd^{2+}or Pb^{2+}treatment increased lignin content, while in lupine the effect was opposite. The decreased lignin accumulation in lupine roots in response to heavy metals, despite an increased PAL activity, suggests that the activated phenylpropanoid pathway was involved in the synthesis of secondary metabolites other than lignin. Kim *et al.* (2013) observed that *C4H* induction was increased for up to 12 h after treatment, followed by a decrease at 24 h after treatment, and a subsequent increase at 48 h after treatment at 150-200 mM NaCl treatment in kenaf seedlings. One of the cDNAs, *C4H2*, is expressed constitutively and seems to play the role of a 'housekeeping' gene in the phenylpropanoid pathway. Conversely, transcripts of *C4H1* could not be detected without induction by wounding of flavedo/leaf tissue; this isoform is likely to be active in wound-induced responses. Expression studies using competitive RT-PCR showed *C4H1* to be strongly wound-inducible, but even in wounded tissue reaching much lower levels of mRNA than *C4H2*. Therefore, *C4H1* may only be able to play its distinctive role when being expressed in specific developmental stages and/or compartments of the cell. *C4H2* is expressed 3–10 times higher than wound-induced *C4H1* even in the control sample (Betz *et al.,* 2001). In this present investigation also more fold increase is found for *C4H2* than *C4H1*. The biosynthesis of lignin and its functional significance during water stress was studied in the leaves of *Trifolium repens* plants subjected to 28 days of drought (Lee *et al.,* 2007). The activities of enzymes involved in the biosynthesis of lignin revealed that the enzyme responses to drought might differ, depending on the period for which plants are exposed to drought. Importance of the phenylpropanoid pathway is not only for lignin production, but also for differentiation and stress responses as observed by Betz *et al.* (2001). The known broad range of secondary compounds in plants produced via the phenylpropanoid pathways, ranging from lignin to specific phytoalexins and differential expression of *C4H* isoforms might reflect the specific need for certain compounds at a certain time or compartment. Up regulation of genes in the multi branched phenylpropanoid pathway leads to accumulation of toxic metabolites such as

phytoalexins and anti-microbial compounds including pathogenesis-related proteins and cell wall components such as hydroxyproline-rich glycoproteins, lignin and its precursors (Zabala *et al.,* 2006). The co-ordinated increase in *C4H* and *PAL* gene expression may participate in the reinforcement of cell walls by increased deposition of the lignin building units (monolignols) and coumarates in the stressed plants and thus the restriction of pathogen invasion (Jadhav *et al.,* 2013).

CONCLUSION

A critical role of phenol was deduced to contribute to the castor drought stress tolerance. The biochemical and molecular analysis of genes, enzymes and products of the phenylpropanoid pathway helped to better understand the molecular mechanism of the castor defense response to drought stress. From the present study it may be concluded that phenylpropanoid pathway is an important biochemical pathway that leads to drought stress tolerance in castor. Further characterization, using over expression or silencing of these genes may confirm their role in drought stress tolerance in castor. Moreover, identification of transcription factors affecting the phenylpropanoid pathway and lignin biosynthesis and deposition will provide new insights into the control of plant development and drought stress tolerance.

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