INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0048/2018/20–9–1929–1936
DOI: 10.17957/IJAB/15.0711
http://www.fspublishers.org

Full Length Article

Morphological and Biochemical Responses of *Jatropha curcas* under Osmotic Stress

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Abstract

The aim of this study was to elucidate the effect of water stress on morphological and biochemical aspects of *Jatropha curcas* L. under *in vitro* conditions (germination, early growth of seedlings and callus cultures) and on soil-grown five months old plants. Different sorbitol treatment levels (0, 0.05, 0.1, 0.15, 0.20, 0.25, 0.3, 0.35, 0.40, 0.45 and 0.5 *M*) were supplemented to MS medium in order to maintain the osmotic stress. Five-month-old pot-grown greenhouse plants were subjected to 100, 75, 50, 25 and 0% field capacities. Results show that increased osmotic stress inhibited germination and seedling growth as indicated by reduction in fresh/dry weights and seedling lengths and enhanced SOD and POX activities. Callus cultures were least influenced physiologically and biochemically with reduction in fresh weight/water content and slight enhancement in soluble protein and peroxidase activity only at higher levels of osmotic stress. Lowest field capacity (0%) decreased fresh weight and increased soluble protein content of soil-grown plant leaves. SOD activity was decreased up to 50% field capacity and then increased in further lower field capacities while peroxidase activity remained unaffected. Hence it can be concluded that both the germination and early seedling growth of *J. curcus* are influenced by water stress considerably. Effect of water stress on callus cultures and mature plants were not that prominent though.

Keywords: *Jatropha curcas*; Osmotic stress; *In vitro* germination; Callus cultures

Introduction

Jatropha curcas L., a multipurpose shrub belongs to family Euphorbiaceae. It is native to South America but now is widely distributed in many parts of the world (Cano-Asseleih *et al.*, 1989). It is an eco-friendly plant as its plantation on waste land can reduce soil erosion and make it fertile. Its seeds contain 30–40% oil whose chemical specifications match with those of international biodiesel standards thus making it an important crop for the production of biofuel (Azam *et al.*, 2005; Martínez-Herrera *et al*., 2006; Kheira and Atta, 2009). It can potentially resolve energy shortage problems, reduce carbon emissions and can increase the income of farmers (Martin and Mayeux, 1985; Banerji *et al*., 1985; Gubitz *et al*., 1999; Keith, 2000; Zhou *et al*., 2006). No wonder that its cultivation is becoming more popular all over the world.

Plants respond to various stresses by certain physiological and developmental changes (Kazuo and Kazuko, 1996), for instance germination in *Jatropha* was shown to be reduced under various abiotic stresses (Shakirova and Sahabutdinove, 2003). Soil water shortage or drought stress (also synonymously referred to as osmotic stress in the literature) can also reduce the plant growth and yield (Boyer, 1982). However, there are contrasting views about drought tolerance level of *J. curcas. S*ome workers considered it to be a drought and salinity tolerant plant species capable of growing in areas with limited availability of water without any significant effect on its growth and physiology (Maes *et al*., 2009; Silva *et al*., 2010; Sapeta *et al*., 2013; Hishida *et al*., 2014). Others reported a significant effect on the yield of *J. curcas* with fluctuations in available amount of water (Singh and Saxena, 2010; Rao *et al.*, 2012; Phiwngam *et al*., 2016). However, most of the experiments for studying drought tolerance have earlier been performed in greenhouse with controlled watering to develop water deficit conditions (Gimeno *et al*., 2012; Niu *et al*., 2012; Santos *et al*., 2013). To the best of our knowledge, studies on early seedling growth of *J. curcas* in *in vitro* conditions under water stress are limited.

Water stress can create oxidative damage which results in an increased generation of reactive oxygen species (Guerfel *et al.*, 2009). Many plant species have developed multiple photo-protective and antioxidative mechanisms to overcome the oxidative stress induced by drought. That is why responses of plants to various stresses are usually determined by studying certain biochemical parameters like protein content and stress-related antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) etc. Changes in quality and quantity of proteins under salt stress in *J. curcas* callus cultures were observed by

To cite this paper: Rizwan, S. and F. Aftab, 2018. Morphological and biochemical responses of *Jatropha curcas* under osmotic stress. *Int. J. Agric. Biol*., 20: 1929–1936

Kumar *et al*. (2008). Later over-expression of a ribosome inactivating protein (JcNAC1) responsible for osmotic, salt and pathogenic stress tolerance in *J. curcas* was reported by Qin *et al*. (2014). Lama *et al*. (2016) have also reported that drought causes oxidative stress in *J. curcas*. However, information regarding the extent of drought tolerance in quantitative terms at various developmental stages of *J. curcas* is still limited.

Different osmotic agents like sorbitol, manitol and polyethylene glycol (PEG) have already been used by various workers to induce osmotic stress in *in vitro* culture media (Almansouri *et al*., 2001; Frank *et al*., 2005; Wang *et al*., 2011a). Polyethylene glycol (PEG) has mostly been used in such studies on *J. curcas* (Qin *et al*., 2005; Silva *et al*., 2010; Wang *et al*., 2011a, b; Qin *et al*., 2014). However, reports on manitol and sorbitol being used as a drought inducing substance for *J. curcas* are scanty. Sorbitol is metabolically more inert than other saccharides and is considered as a non-metabolite (Lambers *et al*., 1981). Addition of sorbitol in MS medium decreased the water potential of the medium thus inducing water stress or osmotic stress (Abu-Romman, 2010). In a similar manner, response of callus cultures and/or whole plants to various abiotic stresses have also been studied and compared for many plant species (Smith and McComb, 1981; Rus *et al*., 1999; Wang *et al*., 1999; Al-ka'aby and Abdul-Qadir, 2011). There is hardly any such study involving *J. curcas* though its growth on marginalized, water deficit soil is reported.

The present study was planned to evaluate water stress tolerance of *J. curcas* by employing partial biochemical characterization involving soluble protein content and activities of potential antioxidant enzymes like SOD and POX. The experiments were performed both at the tissue level using callus cultures and at whole plant level under *in vitro* and pot conditions under different water stress levels. This information will enable us not only to better understand general mechanism for drought tolerance in *J. curcas* but may also be of applied significance in future endeavors involving *J. curcas*'s propagation at mass scale on marginal lands.

Materials and Methods

In vitro Seed Germination and Early Growth of Seedlings

Seeds were washed thoroughly with house-hold detergent (Lemon max Unilever Karachi, Pakistan) adding to it 2–3 drops of Tween 20 (Daejung Chemicals) for 15–20 min at 60ºC and rinsed several times with tap water. Two different chemicals were used to remove all traces of contaminations from hard seed coats. Seeds were then treated with 0.1% mercuric chloride ($HgCl_2$) for 10 min, rinsed 4–5 times with autoclaved distilled water and then with 20% sodium hypochlorite (NaClO) for 20 min and rinsed again 4–5 times with autoclaved distilled water in Laminar Airflow Cabinet (ESCO, Singapore; Model 1750). Seed coats were removed after sterilization and naked seeds were inoculated in culture tubes (30×200 mm) containing 25–30 mL of medium. *In vitro* germination of seeds was carried out on MS medium (Murashige and Skoog, 1962) with 3% sucrose, pH adjusted to 5.8 and solidified with 8% Agar (Sigma Aldrich) without any growth regulators. Cultures were kept in dark at 25±2ºC. After shoot emergence, cultures were shifted to 16/8 h photoperiod for chlorophyll development. Drought stress was induced in culture medium using different sorbitol treatments (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 *M*). Each treatment comprised of 30 culture tubes and experiment was repeated thrice. Germination percentage was measured by number of seeds from which radicle emerged out to develop root out of total number of seeds. Rate of germination was observed after 2, 4, 6 and 10 days of inoculation. Germination energy was calculated by the method proposed by Afzal *et al*. (2017). It was calculated by counting the number of germinated seedlings at fourth days after the start of germination. Data on root/shoot length, fresh/dry weight and biochemical parameters were collected after 10 days of inoculation.

Callus Induction

Callus cultures were established using young leaves (2nd or third leaf from tip) freshly excised from 2–3 year-old plant of *J. curcas*. Leaves were surface sterilized using the same procedure as that for seeds with few modifications, like washing with only household detergent (Tween 20 and high temperature (60ºC) were not applied), treatment with $HgCl_2$ for 5 min, and with NaOCl for 7 min. The sterilized leaf explants were cut into small pieces (3–5 mm) and incubated in culture tubes (25×150 mm) containing 10–15 mL of MS medium supplemented with 3% sucrose, 5 mg/L BAP and 1 mg/L NAA adjusted to pH 5.8±0.2 and solidified with agar (8%) for callus induction. All process of sterilization and inoculation was performed using Laminar Airflow Cabinet. Cultures were kept in 16/8 h photoperiod at a temperature of 25 ± 2ºC. Osmotic stress was induced in culture medium using different Sorbitol treatments i.e., 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 *M*. Each treatment was given in 30 culture tubes and experiments were repeated thrice. Fresh/dry weight, water contents and biochemical attributes were measured after 30 days of inoculation. Water contents were determined by subtracting the dry weight from fresh weight.

Plants Propagated from Stem Cuttings

Stem cuttings of *J. curcas* plants were obtained from *Jatropha* Pakistan Four Friends Group Multan and were sown in pots (14" × 16") containing an equal amount (by volume) of a mixture of sand, soil and peat (1:1:1) during the month of May. Pots were irrigated regularly on weekly basis until the plants reached the age of four months. These plants were then shifted to a greenhouse covered with

polythene sheet (at a height of approximately 14′) one week before exposing to different levels of water stress.

A wide range of water availability was developed (in groups) for these plants. One group of plants was irrigated with measured amount of water to the extent that water started leaking from the bottom of the pot. That was considered at 100% field capacity and amount of water given was noted precisely. Second group of plants with 75% field capacity was irrigated with ¾ the amount of water given to the first group of plants. Likewise third group with 50% field capacity was given ½ and fourth group with 25% field capacity with ¼ amount of that water. Field capacities were maintained by irrigating the pots daily using the same methodology. The fifth group with 0% field capacity was not irrigated at all during the experiment period. Each experimental group consisted of three plants. Plants were subjected to this treatment for 30 days (from 3rd September to 3rd October). Fresh weight, dry weight, water content per unit area of leaves and biochemical parameters of leaves were then assessed.

Biochemical Analysis

Weighed amount (1.0 g) of plant material (germinating seedling, callus or leaf) was crushed in pestle and mortar (ice-chilled overnight) with 0.1 g polyvinyl polypyrrolidone and then added 2 mL of 0.1 *M* phosphate buffer (KH_2PO_4 (13.61 g) and K_2HPO_4 (17.42 g) dissolved in water and making final volume one liter). It was then centrifuged at 15,400 g for 15 min at 4ºC. The supernatant so obtained was stored at 0ºC in refrigerator and used for further protein quantification, peroxidase activity and superoxide dismutase estimation.

Protein was estimated following Biuret method proposed by Racusen and Johnstone (1961). The experimental tubes contained biuret reagent (2.0 mL) and extract obtained from above procedure (0.2 mL) while control contained biuret reagent (2.0 mL) and distilled water (0.2 mL) instead of extract. The optical densities of experimental samples were measured spectrophotometrically after 10 min at 560 nm against control which was considered as zero. A standard curve for proteins developed using bovine serum albumin (E Merck Ag Darmstadt Germany) was used to calculate the amount of protein.

For quantitative analysis of peroxidase, modified Guaiacol-H_2O_2 method by Luck (1974) was employed. The assay mixture contained crude enzyme extract (0.1 mL), 0.1 M phosphate buffer (3.0 mL of) (pH 7.2), 20 m*M* guaiacol solution (2-methoxyphenol) (0.05 mL of) and 12.3 m*M* H_2O_2 solution (0.03 mL). The time required to increase the absorbance value by 0.1 at 240 nm was used to calculate the peroxidase activity and expressed as units per milliliter enzyme.

Superoxide dismutase (SOD) activity was estimated by the modified methods established by Maral *et al*. (1977). It was measured in terms of its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT). Two sets of tubes were taken, each containing 2.0 mL of reaction mixture. One set of tubes was experimental containing 5.0 μL enzyme extract added in reaction mixture while the other set of tubes was control contained reaction mixture without the enzyme extract. Both sets of tubes were placed 30 cm below the light of two 30 W fluorescent tubes for 15 min. The absorbance of the sample and control mixtures was measured at 560 nm. SOD activity was expressed as units per milligram of protein.

Statistical Analysis

Statistical analysis of data was performed using ANOVA with the help of computer software (SPSS Version 16). Mean values and the standard errors were calculated for each experiment. Significant differences between the mean values were also compared using Duncan's multiple range tests.

Results

***In vitro* Seed Germination and Early Growth of Seedlings**

After 2 days of *in vitro* germination test, most of the seeds germinated in control and 0.05 *M* sorbitol treatments while no remarkable germination was observed at higher concentrations. With the passage of time, seeds also germinated in still higher concentrations. However, it is also clear from the data that after 10 days, sorbitol treatments above 0.15 *M* significantly affected percentage germination which reduced to zero above 0.3 *M* sorbitol (Fig. 1a). Germination energy was also reduced significantly with increasing osmotic stress (Fig. 1b).

Fresh, dry weight and shoot length decreased with an increase in sorbitol treatments while root length was not significantly affected up to 0.1 *M* and then afterwards decreased in higher sorbitol treatments (Fig. 2a, 2b and Fig. 6). There was no significant effect on soluble proteins with increase in sorbitol treatment as compared to control (Fig. 3c). Peroxidase and SOD activities of *in vitro* germinating seedlings increased with increase in sorbitol concentration as compared to control (Fig. 3a and b).

Callus Cultures

Dry weight of callus cultures was not significantly influenced with increase in sorbitol treatments. Fresh weight and water contents of callus cultures first increased in lower sorbitol concentrations (0.05 *M* and 0.1 *M*) as compared to control. No significant effect on these parameters was observed in higher treatments up to 0.45 *M*. However, decrease in these parameters was observed at the highest sorbitol treatment (0.50 *M*) (Fig. 4a and 4b).

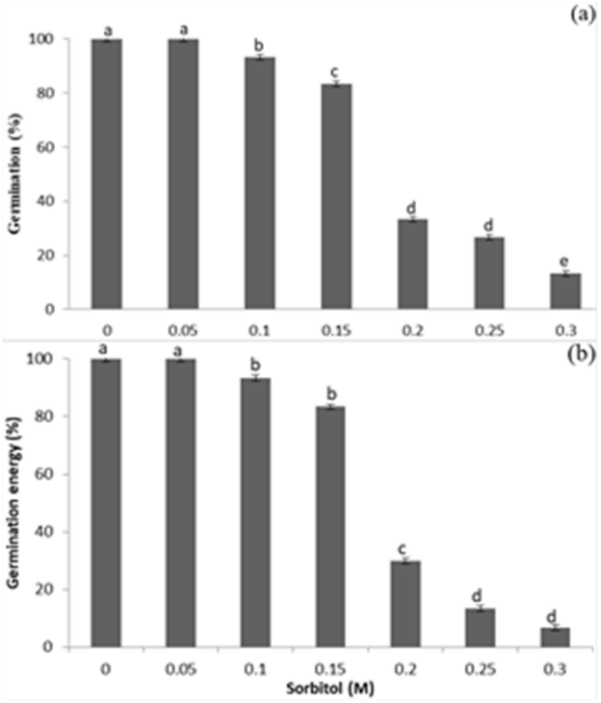

Fig. 1: Effect of different sorbitol concentrations on *in vitro* germination of *J. curcas* seeds

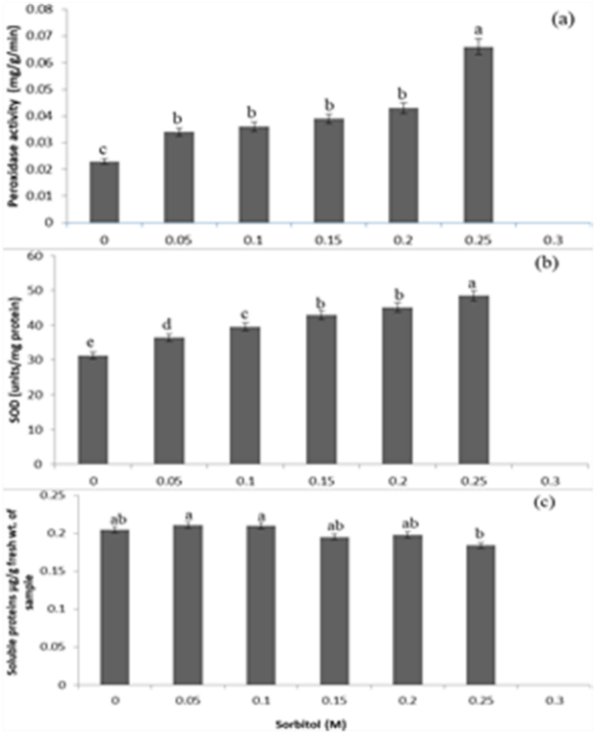

Fig. 3: Effect of different sorbitol concentrations on biochemical parameters of *in vitro* seedlings of *J. curcas*

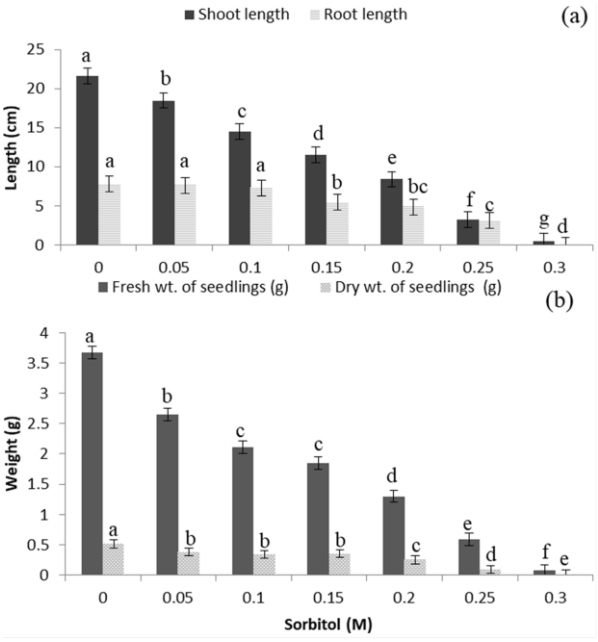

Fig. 2: Effect of different sorbitol concentrations on seedling length and weight of *in vitro* seedlings of *J. curcas*

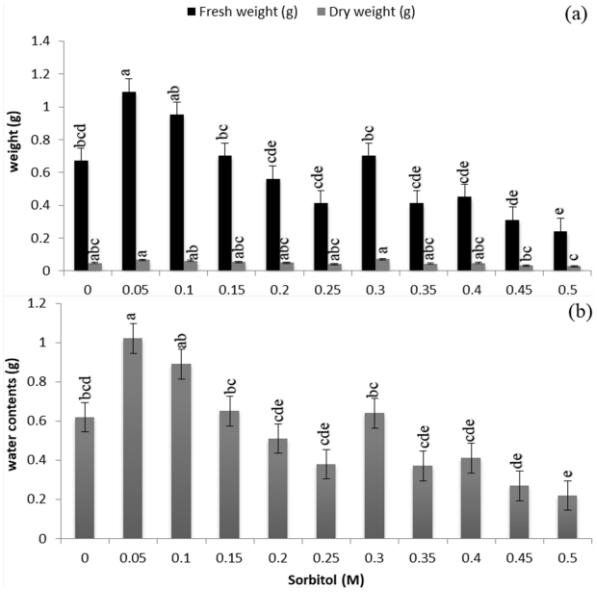

Fig. 4: Effect of different sorbitol concentrations on fresh/dry weights and water contents of callus cultures derived from *J. curcas* leaf explant

There was a slight reduction in soluble protein contents with increase in osmotic potential up to 0.2 *M* that then started increasing above this osmotic potential and maximum soluble protein was detected in calluses grown on highest osmotic potential (0.5 *M*) as shown in Fig. 5c. There was no significant effect on peroxidase activity shown by callus cultures up to 0.30 *M* sorbitol and then it increased with increase in sorbitol concentration. Maximum

peroxidase activity was observed in 0.5 *M* sorbitol (Fig. 5a). Osmotic stress did not show any remarkable effect on SOD activity of callus cultures (Fig. 5b).

Plants Propagated from Stem Cuttings

There were no visual symptoms of stress in these plants irrespective of different field capacities of water. Only the plants that were not watered at all (0% F.C.) showed slight decrease in fresh weight of leaves per unit area. Maximum fresh/dry weight and water contents per unit area of leaves were exhibited by plants with 50% F.C. as shown in Fig. 5. There was no effect observed on dry weight per unit area of leaves in different field capacities.

Maximum soluble proteins were detected in leaves that were not watered at all during the experimental period (0% field capacity). There was no significant effect on peroxidase activity shown by leaves at all levels of applied water stress. Minimum SOD activity was in *Jatropha* leaves at 50% field capacity and there was trend towards increase in SOD activity both in lower (0 and 25%) and higher field capacities (75 and 100%) as shown in Table 1.

Discussion

It is a well-known fact that the plants resist abiotic stresses through changes in the morphological characters along with altered biochemical, and metabolic processes (Ingram and Bartels, 1996; Lokhande *et al*., 2010). In this study, water stress had shown a significant effect on germination as well as early seedling development during *in vitro* germination trials. However, callus induction, its proliferation, and 5 month old plants in pots did not exhibit any remarkable growth effect under similar set of conditions.

Reduced germination of *Jatropha* as well as its delayed onset by an increase in osmotic stress under *in vitro* conditions was observed in our experiments. These results are in line with Singh and Saxena (2010) who observed similar results for *J. curcas* seed germination under field conditions. Decreased germination of *J. curcas* seeds by different abiotic stresses was also reported previously by several workers (Shakirova and Sahabutdinove, 2003; Windauer *et al*., 2012; Senger *et al*., 2014). Osmotic stress results in reduced supply of water due to low soil water potential, which hinders imbibitional process, and in turn germination. Reduced germination under osmotic stress or water stress may also be due to the fact that seeds develop an osmotically enforced dormancy that may be an adaptive strategy under stressful conditions (Singh *et al*., 1996). However at the same time, reduction in water contents of embryos and endosperms under osmotic stress was observed by several workers in different plants (Siddique *et al*., 2000; Gill *et al*., 2001). Similarly fresh/dry weight and shoot/root length of *in vitro* germinating seedlings also reduced significantly with an increase in osmotic stress. This might be due to the reason that amongst

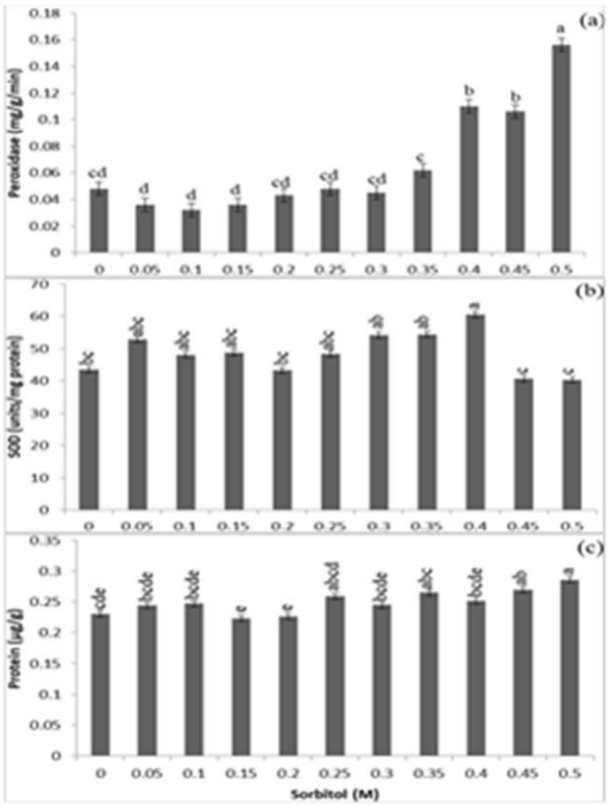

Fig. 5: Effect of different sorbitol concentrations on biochemical parameters of callus cultures of *Jatropha curcas*

Fig. 6: Effect of different Sorbitol concentrations on physical appearance of *in vitro* germinating seedlings of *Jatropha curcas*

the most sensitive stages in the life cycle of many plants to environmental stresses are seed germination and early growth of seedlings (Jones, 1986; Li *et al*., 2011). Reduction in early growth of seedlings with increased osmotic stress may be due to an inhibitory effect of water stress on cell elongation due to decreased water contents in the tissues

Table 1: Effect of different field capacities of water in the pot soil on physical and biochemical parameters of *Jatropha curcas* leaves after one month of treatment

Treatments (% Field Capacity)	Fresh weight/leaf unit area (g)[A]	Dry weight/unit area (g)[A]	Water content/unit area (g)[A]	Peroxidase activity[A] (mg/g/min)	SOD activity[A]	Soluble proteins[A] μg/g fresh wt. of sample
0	2.77±0.067[b]	0.497±0.012[b]	2.27±0.07[b]	0.062±0.003[a]	60.72±0.43[ab]	0.214±0.002[a]
25	2.97±0.067[ab]	0.517±0.019[ab]	2.45±0.06[ab]	0.051±0.01[a]	59.64±0.72[b]	0.181±0.013[b]
50	3.13±0.037[a]	0.530±0.026[ab]	2.59±0.01[a]	0.079±0.006[a]	48.62±1.16[d]	0.166±0.006[bc]
75	3.00±0.069[a]	0.567±0.02[a]	2.44±0.05[ab]	0.077±0.02[a]	54.07±1.63[c]	0.142±0.005[c]
100	2.99±0.087[a]	0.540±0.017[ab]	2.45±0.07[ab]	0.072±0.004[a]	62.99±0.19[a]	0.185±0.011[b]

[A] Data presented here are means of 6 values per treatment

Different letters within a column represent significant difference at P= 0.05 according to Duncan's multiple range tests

(Taiz and Zeiger, 2006). Under drought stress, cell expansion and growth is suppressed due to the loss of turgor pressure or osmotic imbalance which in turn reduces the growth and metabolic activity of the plants and finally leads to their death (Jaleel *et al*., 2008). Previously reduction in growth of different plants (including *J. curcas*) due to drought stress was also reported by some workers (Nemeth *et al*., 2002; Yin *et al.*, 2010).

Increase in callus fresh/dry weight and water contents at lower sorbitol concentrations may be due to increased accumulation of carbohydrates for turgor maintenance as also reported by Javed and Ikram (2008). Newton *et al*. (1987) suggested that plants that cannot maintain turgor pressure under enhanced stress conditions are unable to grow and maintain growth. Here, calluses of *J. curcas* maintained their growth under osmotic stress indicating that they probably have mechanism developed to combat stress conditions like enhanced activities of antioxidant enzymes, production of stress proteins etc. (Munir and Aftab, 2013; Ikeuchi *et al*., 2013;). These mechanisms have also been investigated and seem to be working in the current study as well.

Leaves from five-month-old plants with water deficit treatment did not show any visual symptoms of stress like necrosis, chlorosis or even drying. These results are in line with the study of Silva *et al*. (2012) suggesting that developed *J. curcas* plants could withstand drought conditions. Estimation of fresh/dry weight per unit area of leaves also indicated these plants as drought tolerant. This tolerance to stress may be due to the expression of certain plant-specific transcription factors as JcNAC1 which was reported in *J. curcas* when exposed to abiotic stress by Qin *et al.* (2014). However, Arcoverde *et al.* (2011) have reported that under severe water stress, leaf relative water content (RWC) was moderately reduced in *J. curcas*. Significant decrease in water contents of leaves at 0% field capacity was also observed presently.

In *J. curcas*, enhanced activities of SOD and peroxidases under drought stress were reported by Silva *et al.* (2012). Similar results were observed in the present experiments where *in vitro* germinating seedlings and soil-grown plant leaves showed increased SOD and peroxidase activities under stress conditions. Higher water capacities (75% and 100%) were also proved to be stressful for *J. curcas* plants as there was also an increase in SOD activities as compared to moderate water supplies (50%). Previously, Pompelli *et al*. (2010) also reported that CAT, APX, SOD activities and GS, MDA contents in *J. curcas* leaves were enhanced significantly in the water-stressed conditions as compared to well-watered conditions. These observations give us clear idea that water stress creates oxidative stress in *Jatropha* that resulted in enhanced production of antioxidant enzymes to minimize the oxidative damage. These antioxidant enzymes may play an important role to enhance plants' capability to survive in the water deficit conditions. SOD activities remained unaffected in callus cultures with an increase in osmotic stress however; peroxidase activity was enhanced above 0.3 *M* sorbitol treatment. This is perhaps because apart from SOD and peroxidase activities, xanthophyll cycle, photorespiration and antioxidant enzymes found in peroxisomes and chloroplasts also play role to prevent oxidative damage (Coue´e *et al.*, 2006). These parameters could be analyzed in future studies. This however indicates that callus cultures might show different behavior as compared to *in vitro* seedlings. Neumann (1997) also reported such contrasting behavior by concluding that isolated cells were much more tolerant to osmotic stress as compared to the whole plant.

Increase in protein content of seedlings at 0.05 *M* and 0.1 *M* sorbitol as compared to control (0 *M*) may be due to the production of stress induced proteins (Cherian and Reddy, 2003) while decrease in soluble protein content at higher osmotic potential may be due to decreased production of proteins under stress conditions (Vogel *et al.*, 2011). Same is the case with callus cultures where there was a significant increase in soluble proteins at 0.05 *M* and 0.1 *M* sorbitol concentrations and then decreased with further increase in osmotic stress. Increased production of soluble proteins in *Jatropha* leaves that were not watered at all might be due to the production of stress proteins. Some stress proteins produced as a result of stress applied to plants are also reported by some workers in *J. curcas* (Qin *et al*., 2005; Zhang *et al*., 2007).

Callus cultures are mostly used in experiments for studying physical and biochemical parameters as the response of individual cell is considered equivalent to the whole plant. We may say that while the early seedling growth was affected by drought stress, mature plants would have developed mechanism for combating the stress conditions. Callus cultures are developed from young leaves

of matured plants and their responses to osmotic stress are almost the same as those of matured plants. Hence the mechanism of stress tolerance perhaps works at cellular level in *J. curcas.*

Conclusion

Sorbitol-induced osmotic stress has reduced as well as delayed germination of *J. curcas* seeds. At the same time seedlings also showed sensitivity to osmotic stress as it is evident from reduced growth and enhanced antioxidant enzyme activities. Later, callus cultures developed under different osmotic stress conditions from leaves of mature plants did not show any significant effect on their growth. Our results indicated that five months old pot plants were tolerant to water stress, though exhibited better growth and development with moderate water supply (50 and 75% F.C). Callus cultures were also stress tolerant as they responded in almost same manner as mature intact plants. Hence the use of callus cultures could be suggested for further investigations of *J. curcas* under abiotic stresses. From this study, it could be suggested that if provided with adequate amount of water for germination and early seedling growth, *J. curcas* may be propagated in marginal wastelands in future.

Acknowledgements

The authors feel grateful to Higher Education Commission Islamabad for proving financial aid in the form of Indigenous 5000 Fellowship Program Batch 3 (PIN No. 063-121479 Bm 3-027) during the present research work.

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(Received 11 January 2018; Accepted 14 April 2018)