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Salicylic Acid and Acquisition of Desiccation Tolerance in *Pisum sativum* Seeds

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Abstract: Desiccation tolerance in orthodox seeds is acquired during various phases of development and lost upon imbibition/germination. In this study, germinated seeds of pea (*Pisum sativum*) with a protruded radicle length of 10 mm did not survive desiccation below 0.43 g H_2O g^{-1}. The re-establishment/resumption of radicle growth after desiccation were achieved under both Mili-Q water and salicylic acid (150 μM) re-growth media, separately. Capacity to resume desiccation tolerance after the process of germination was limited to a period of growth characterized by radicle lengths between 5 and 10 mm. After silica gel drying (1 and 6 h), radicles of pea performed far better in terms of re-growth under salicylic acid media, than the Mili-Q water. Further, the levels of reactive oxygen species was measured to be high (3.2 fold) in Mili-Q water regrown radicles compared to salicylic acid. Other side, higher magnitudes of both protein (1.2 fold) and DNA (1.7 fold) were measured in salicylic acid grown radicles but contained low levels (1.7 fold) of oxidized protein than the Mili-Q water sample. Even after putting huge efforts, we are failed to detect traces of fragmentation/DNA oxidation possibly due to the exerted level of stress was insufficient to induce any change in it. Results showed that exogenous supply of salicylic acid accelerates the desiccation tolerance acquisition capacity in germinated pea seeds/radicles by maintaining low magnitude of free radicals at one end and boosting amounts of both protein and DNA at the other.

Key words: Desiccation tolerance, germination, *Pisum sativum*, radicle length, water content

INTRODUCTION

Desiccation Tolerance (DT) can be conceptually defined as the capacity to survive after the removal of almost all (80-90%) of cellular water in equilibrium with moderately dry air and can resume normal metabolism when re-hydrated (Oliver *et al*., 2000; Buitink *et al*., 2006). By definition, DT is the capability of any living organism to deal with water losses below 0.3 g H_2O g^{-1} dry mass and survival after rehydration without permanent damage (Oliver *et al*., 2000). Under such conditions there is no free water in the living cells and the structure of macromolecules and membranes are protected by specific mechanisms (Oliver *et al*., 2000; Hoekstra *et al*., 2001). This phenomenon is widespread across the plant kingdom including ferns, mosses, pollens, angiosperms, resurrection plants and seeds of orthodox category. Bewley (1979) has argued that three protoplasmic properties are immensely needed to establish DT in plants or its parts: (1) Minimize damage from dehydration and/or rehydration, (2) Retain cellular integrity even in the dry state and (3) Mobilize repair/correction mechanisms upon rehydration. These criterions give emphasis to two very important processes: Protection and repair which must arise during acquisition of DT (Oliver *et al*., 2005). Several precise mechanisms have been implicated in acquisition and maintenance of DT, including: Reduction in the extent of vacuolization, intracellular de-differentiation, 'switching-off' of metabolism, accumulation of protective molecules viz., Late Embryogenic Abundant (LEA) proteins, sucrose and certain oligosaccharides and efficient operation of antioxidant system (Hoekstra *et al*., 2001). Among these, polysaccharides and LEA are shown to stabilize membranes and macromolecules during desiccation and facilitating water shell to them (Hoekstra *et al*., 2001). Length of protruded radicle and water potential of its cells are the key determinants affecting re-induction of DT. Although, repair mechanisms (antioxidant enzymes) have been identified in mosses and resurrection plants (Oliver *et al*., 2005) their exact function is still to resolve or not proven in seeds DT.

Findings concerning to acquisition of DT during seed development and its loss during germination have already been reported in past (Faria *et al*., 2005). Interestingly, DT can be rescued in germinated seeds applying a mild osmotic shock and studies have also been conducted to resolve the mechanisms of DT in germinated

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orthodox seeds (Faria *et al*., 2005; Buitink *et al*., 2006). In line, DT capacities in germinated seeds of *Cucumis sativus* and *Impatiens walleriana* were restored fully after their exposure to PEG (Bruggink and Van der Toorn, 1995).

When seeds are imbibed and then desiccated, rate of survival and/or DT capacity declined gradually with increasing incubation time (Faria *et al*., 2005). Thus, germinating orthodox seeds have resemblance with recalcitrants. Therefore, it has been recommended to be a popular model for exploring the mechanisms of DT (Sun, 1999). It was well recognized that during germination, Reactive Oxygen Species (ROS) are produced in excess which can be one of the sources of damage during tissue dehydration. In general, extra availability of ROS was shown to be the very first response of desiccation which are known to react readily with macromolecules and disrupts normal cellular functioning (Parkhey *et al*., 2012; Keshavkant *et al*., 2012). Further, seed also suffers oxidative stress during maturation drying phase (just before shedding from mother plant) of its development where proteins and DNA can possibly be affected deleteriously leading loss of vigour and then viability (Osborne *et al*., 2002).

Salicylic Acid (SA) is well known for its ability to confer tolerance therefore extensively used as protective agent against variety of biotic and abiotic stresses including desiccation (Hayat *et al*., 2012). Its beneficial effects on detoxification of ROS and upregulation of antioxidant potential are also being published (Hayat *et al*., 2012). Salicylic Acid (SA) is proven to have growth boosting property which is conferred by their ability to alter balance between hormones like; Gibberellic Acid (GA), abscisic acid (ABA) and cytokinins (Hayat *et al*., 2010). Salicylic Acid (SA) is proven to induce varied responses in plants viz., stimulated root/shoot growth, protein synthesis, increased cytokinin synthesis, transient rise in ABA, accumulation of protective substances, etc. (Hayat *et al*., 2010, 2012).

Thus, exogenous supply of SA to desiccation intolerant stage of pea seed/radicle is supposed to enhance or re-induce DT. Keeping this approach, present study was designed to investigate the relationship of ROS with the loss and re-induction of DT in germinated pea seeds. Additionally, effort has also been invested to explore the contributions of both protein and DNA in conferring DT to germinated pea seeds.

MATERIALS AND METHODS

Plant sample: The pea (*Pisum sativum*) seeds were purchased from the local market. Abnormal and physically damaged seeds were discarded. Healthy and uniform sized seeds were sorted out. Selected seeds were stored at ambient laboratory conditions (Temperature 26-28°C, Relative Humidity 45-50%) and were harvested time to time for various analyses.

Seed germination: The pea seeds were first rinsed thoroughly with Mili-Q water (MW) (Millipore, Gradient A-10, USA) for 1-2 min. There after, surface sterilization was done with sodium hypochlorite solution (1%, v/v) and rinsed them thoroughly with MW, to remove traces of sterilization media. These seeds were then placed in germination boxes of fixed size (30×15×5 cm), containing two layers of paper towels, moistened with MW (Parkhey *et al*., 2012). Above boxes were then placed in darkness at 26-28°C and maintained there up to the protrusion of radicles of required lengths. Mili-Q water (MW) was supplied to the germinating seeds whenever needed. Germinated seeds exhibiting radicle lengths of 1, 5, 10 and 15 mm, were harvested time to time and their radicles were removed using a sharp razor for further experimental usage.

Desiccation trials: The self indicating silica gels of standard quality were purchased from the scientific store and then kept in an oven for 12-15 h at 70-80°C for its activation. Activated silica gels were taken out and cooled at ambient temperature. Fresh radicles of pea seeds of required lengths were placed in net bags to protect their mixing with silica. Radicle containing bags were placed over a layer of activated silica, half-filled in a glass desiccator and above this a layer of silica was spread again to extract tissue water evenly from all the sides. Radicles were desiccated for 1 and 6 h separately and hydrated silica was replaced after each hour with fresh lots.

Re-growth of desiccated radicle: Desiccated pea radicles (for 1 and 6 h, separately) of varied lengths were divided into two separate groups; first group was kept for re-growth over paper towels moistened with MW in a petri dish for next 24 h. Second group of radicles were regrown on SA (150 μM) supplemented paper towels, for specified time length. Afterwards, radicles were harvested from the respective growth media and used for further investigations.

Determination of water content: The Water Content (WC) of radicles of pea seed was determined gravimetrically and expressed as g H_2O g^{-1}. Pea radicles of varied lengths were kept in an oven at 103°C for 17 h, in order to determine their WC (Parkhey *et al*., 2014).

Estimation of ROS

Superoxide radical: Superoxide generation was determined following Parkhey *et al.* (2012). Both MW and SA grown radicles of differing lengths were incubated in potassium phosphate buffer (20 mM, pH 6.0), containing XTT (Sodium salt), for 6 h in darkness, at ambient temperature on a shaker. Reduction of XTT salt was measured at 470 nm, using a UV-Vis spectrophotometer (ATI-Unicam, UK). Amount of XTT reduced was calculated using an extinction coefficient of 2.16×10^4 M^{-1} cm^{-1} and expressed as μM $radicle^{-1}$.

Hydrogen peroxide: Radicles of various lengths were incubated in potassium phosphate buffer (20 mM, pH 6.0) consisting 5 μM scopoletin and horseradish peroxidase (3 μg mL^{-1}), on a shaker, for 6 h, at 25°C. Generated H_2O_2 was measured using spectrofluorometer (Shimadzu, Japan) at excitation of 346 nm and emission of 455 nm (Parkhey *et al.*, 2012) and expressed as mM $radicle^{-1}$.

Protein extraction and estimation: Weighed (100 mg) tissues were extracted with cold Zivy's Buffer (0.03 M Tris base, pH 8.5) comprising 10 mM ascorbic acid, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF by using a cold pestle and mortar (Keshavkant *et al.*, 2012). The homogenates were centrifuged at 10,000 rpm for 20 min at 4°C. Supernatant thus obtained is used for different analyses.

Content of protein was measured following Bradford (1976) and was expressed as mg protein per gram Fresh Mass (FM).

Protein oxidation: Oxidized (carbonylated) proteins were assayed following their reactivity with 2,4-dinitrophenyl hydrazine (DNPH) to form hydrazones (Keshavkant *et al.*, 2012). An aliquot (200 μL) of isolated protein was mixed with 10 mM DNPH (300 μL) prepared in 2 M HCl and blank was mixed with 2 M HCl only, allowed the tubes to stand for 1 h at ambient temperature. Proteins precipitated with TCA (10%, w/v) were washed twice with ethanol:ethyl acetate (1:1, v/v) mixture. Washed pellets were then dissolved in 6 M guanidine hydrochloride (2 mL, prepared in potassium phosphate buffer; 20 mM of pH 2.3). After recording absorbance of the product at 370 nm, actual carbonyl content was calculated following a molar absorption coefficient of 22000 M^{-1} cm^{-1} and expressed as mM g^{-1} protein.

DNA isolation and estimation: To isolate genomic DNA, crushed tissue (0.2 g) was homogenized with extraction media (0.1 M Tris of pH 8.0, having 0.05 M EDTA, 1.5 M NaCl, 4% (w/v) CTAB, 1% (w/v) polyvinyl pyrollidon and 1% (v/v) β-mercaptoethanol). Homogenate was incubated at 65°C for 1 h with intermittent shaking and then cooled. Sample was mixed with 250 μL of potassium acetate (5 M) and kept in ice bath for 30 min. Tube was centrifuged at 10,000 rpm for 10 min at 4°C and collected the supernatant. This precipitation step was repeated twice to eliminate interfering compounds. Supernatant was mixed properly with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), allowed to stand for 30 min at ambient temperature and centrifuged (10,000 rpm for 10 min). Above step was repeated thrice to remove lipids, proteins and polysaccharides completely. Supernatant thus obtained was firstly mixed with equal volume of chilled isopropanol and then kept overnight at -20°C. Afterwards, sample was centrifuged at 10,000 rpm for 10 min at 4°C and pellet was washed carefully with 70% ethanol twice. Finally, pellet was dissolved in TE buffer of pH 8.0 (Ray *et al.*, 1996).

An aliquot (20 μL) of isolated DNA was mixed with 980 μL of 0.5 N perchloric acid and 2 mL of Burton's Reagent (Ray *et al.*, 1996). After 16 h of incubation in dark at ambient temperature, absorbance of the complex was measured at 600 nm and content of DNA was expressed as mg g^{-1} FM.

DNA oxidation: Isolated DNA (20 μL) was mixed with 2 mL of 0.6% (w/v) 2-thiobarbituric acid and allowed to stand at 90°C for 30 min (Langfinger and Von Sonntag, 1985). Absorbance of the red complex was read at 537 nm and DNA oxidation was presented as nM mg^{-1} DNA.

DNA fragmentation: Fragmentation of DNA was assayed after Ray *et al.* (1996). For this, frozen radicle (100 mg) was homogenized with lysis buffer (10 mM Tris-HCl, 20 mM EDTA, 0.5% (v/v) Triton X-100) of pH 8.0. Homogenate was spin down for 20 min at 20,000 rpm to separate intact chromatin in the pellet and fragmented DNA in the supernatant. Both pellet and supernatant were mixed separately with 1 mL of perchloric acid (0.5 N), incubated at 90°C for 20 min and centrifuged (for 10 min at 10,000 rpm). To the supernatants, added Burton's reagent and kept for 16 h in dark, at ambient temperature. Absorbance of the sample was measured at 600 nm and data was published as percentage fragmentation.

Statistical analysis: One way analysis (ANOVA) was performed to check the significance of results.

RESULTS

Germination and radicle formation: Seeds of pea, without any pre-germination treatment, registered 100%

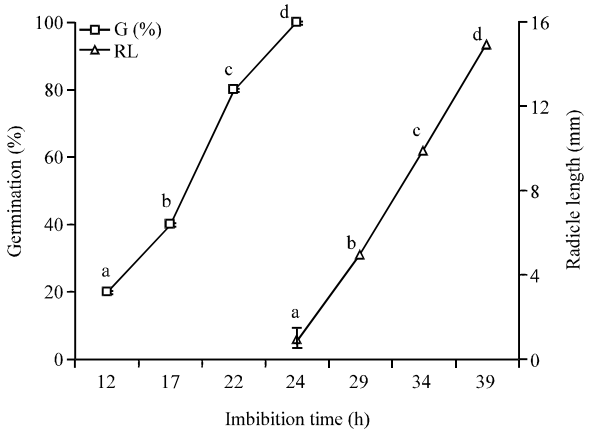

Fig. 1: Germination and increase in radicle length of *Pisum sativum* seeds upon imbibition in Mili-Q water with respect to time. Data presented is means of 5 replicates±SD. At the level of $p<0.05$, letters are showing significant differences

germination after 24 h of incubation (Fig. 1). These seeds exhibited protrusion of radicle (1 mm) after 24 h of soaking in MW. Further, radicles of 5, 10 and 15 mm lengths were obtained after 29, 34 and 39 h of imbibition in MW (Fig. 1).

Water content and desiccation tolerance: Water contents of pea radicles of different lengths were measured in MW and SA regrown samples (Fig. 2a) which exhibited a rising trend of it along with radicle lengths, but irrespective of desiccation timing and re-growth medium. On receipt, WC of freshly harvested radicles of both 1 and 15 mm lengths were 0.53 and 0.60 g H_2O g^{-1}, respectively (Fig. 2a). When these radicles were dried for 1 h and then regrown in MW, they exhibited 0.22 and 0.41 g water g^{-1} and when desiccated for 6 h and then regrown in same media. They contained 0.24 and 0.46 g H_2O g^{-1} of WC (Fig. 2a). Significant difference in WC was noticed between 6 and 1 h desiccated and then MW subjected radicles. Matching observations were noticed with 1 as well as 6 h desiccated and SA regrown radicles of different lengths (Fig. 2a). But a major difference noted is that, SA regrown radicles were having higher values of WC, irrespective of radicle lengths (Fig. 2a).

During further investigation, a regular drop in DT capacity was observed either with increasing radicle length or advancing desiccation. Although, the rate of drop was more in 6 h as compared to 1 h desiccated radicles (Fig. 2b). The 1 h dried and MW regrown radicles of 1 mm length reflected 50% DT. Later, with increasing radicle length, gradual fall in their DT was noted viz.,; for 5 mm: 40%, 10 mm: 30% and 15 mm: 20% only. Whereas,

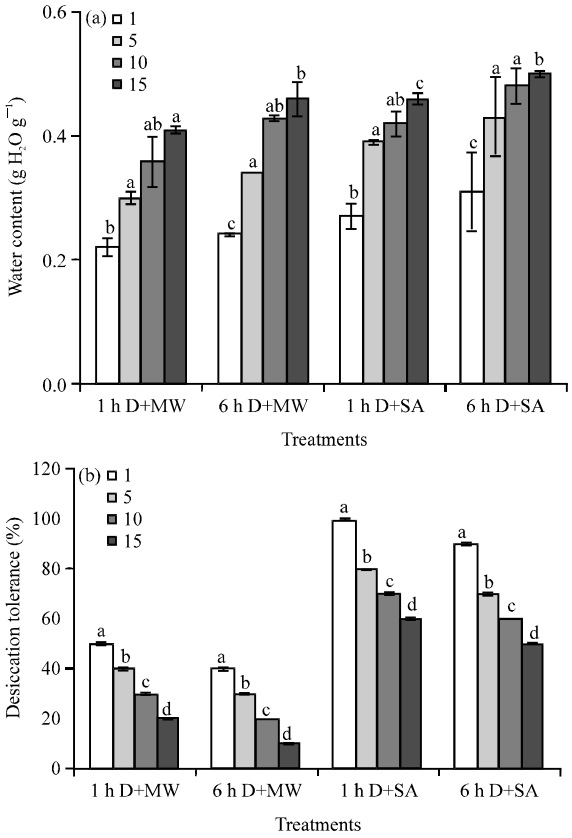

Fig. 2(a-b): Variations in (a) Water content and (b) Desiccation tolerance of *Pisum sativum* seedlings in response to desiccation-rehydration treatments (1 h D+MW/6 h D+MW = Desiccated for 1 and 6 h separately then regrown in Mili-Q water, 1 h D+SA/6 h D+SA = Desiccated for 1 and 6 h separately then regrown in SA). Bars represent mean values of five independent measurements±SD. Letters linked are significantly different at $p<0.05$ level

in 6 h dried, MW incubated radicles, remarkable drop (40% for 1 mm and only 10% for 15 mm radicle) in DT capacity was recorded. Other side, when 1 and 6 h desiccated radicles of varied lengths were regrown in SA, significantly higher (90-100% for 1 mm radicle and 60% for 15 mm radicle) values of DT capacity was measured (Fig. 2b).

Growth performance of desiccated radicles: Growth performance of pea radicle was analyzed after measuring further increments in length (Fig. 3). A significant drop in the elongation capacity was noted particularly in the

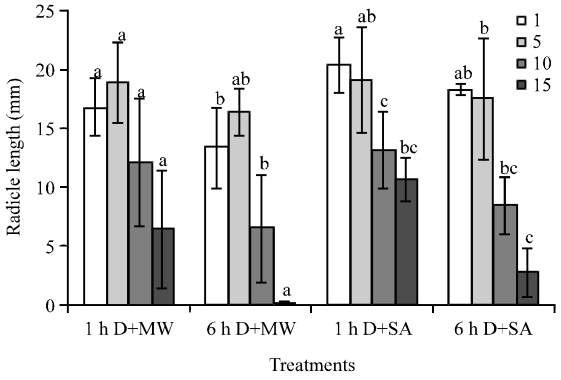

Fig. 3: Fluctuations in length of *Pisum sativum* radicles after desiccation-rehydration treatments (1 h D+MW/6 h D+MW = Desiccated for 1 and 6 h separately then regrown in Mili-Q water, 1 h D+SA/6 h D+SA = Desiccated for 1 and 6 h separately then regrown in SA). Bars represent mean values of five independent replicates±SD. Alphabets associated are significantly different at the level of $p<0.05$

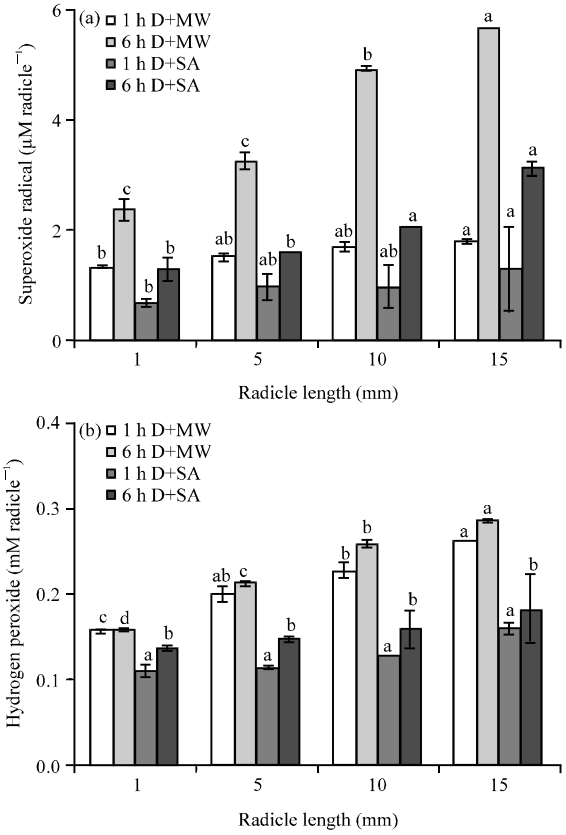

Fig. 4(a-b): Productions of (a) Superoxide and (b) Hydrogen peroxide in *Pisum sativum* seedlings of 1, 5 10 and 15 mm lengths (1 h D+MW/6 h D+MW = Desiccated for 1 and 6 h separately and then regrown in Mili-Q water, 1 h D+SA/6 h D+SA = Desiccated for 1 and 6 h separately and then regrown in SA). Bars represent mean values of five individual observations±SD. Values not related by same letter are significantly different at $p<0.05$ level

larger radicles. Under both MW and SA media, irrespective of desiccation, radicles of 1 mm exhibited maximum increments in length, whereas 15 mm radicles performed least increments (Fig. 3). Additionally, rate of elongation was anti-parallel to increasing radicle length, means fastest in the 1 mm and slowest in case of 15 mm radicle. Further, during comparison of desiccation timing, pace of protrusion decreased only a little in 1 h dried radicles as compared to the 6 h desiccated under both the re-growth media (Fig. 3). Lastly, over the SA media, all the radicles performed far better than the MW. For instance, 1 mm of radicle was grown up to 16.83 and 13.41 mm, after 1 and 6 h of desiccation, respectively, over MW media, but was grown up to 20.42 and 18.33 mm on SA media (Fig. 3).

Reactive oxygen species: In both MW and SA re-grown radicles, accumulations of ROS was registered along with increasing length (Fig. 4a, b). When desiccated radicles of various lengths were subjected to MW, they exhibited elevated levels of superoxide and hydrogen peroxide. Other hand, their contents were at low (significantly), when were regrown in SA media (Fig. 4a, b). Further, significantly higher amounts of both the ROS were investigated in 6 h dried samples of different lengths, compared to 1 h desiccated radicles (Fig. 4a, b).

Protein content: Pea radicles, harvested at different stages of seed germination i.e., 1, 5, 10 and 15 mm lengths, were analyzed for protein content which revealed declining trend with increasing radicle length (Fig. 5a). A slow fall in protein content was noticed after 1 h of desiccation. For example; 1 mm: 15.63, 5 mm: 13.85, 10 mm: 12.81 and 15 mm: 10.72 mg g^{-1} FM but was more pronounced in 6 h desiccated radicles (1 mm: 11.14 and 15 mm: 3.47 mg g^{-1} FM). In contrast, considerably higher amount of protein was determined when dried (1 and 6 h) radicles of various lengths were regrown over SA media (Fig. 5a).

Protein oxidation: Inverse to the protein data, remarkable upsurge in oxidized protein was calculated along with desiccation timing and radicle length (Fig. 5b). Additionally, amount of it reflected increasing trend under

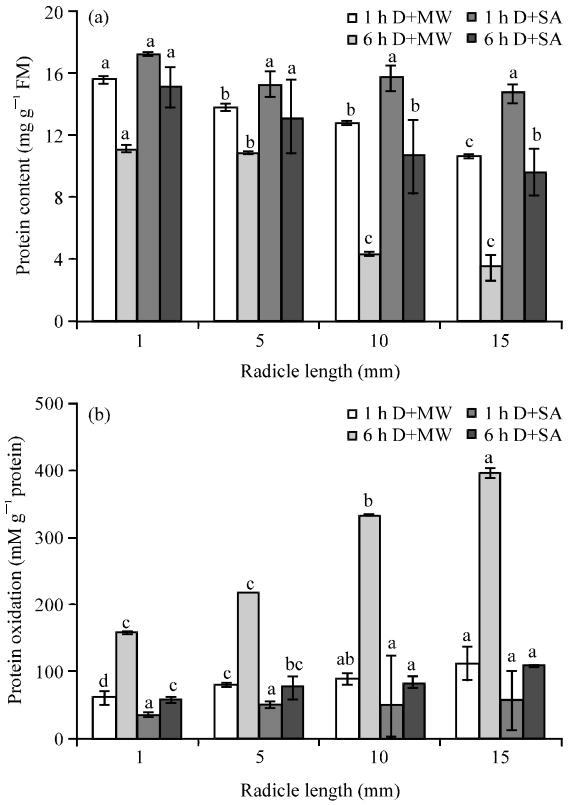

Fig. 5(a-b): Accumulations of (a) Protein and (b) Oxidized protein in desiccation-rehydration treated *Pisum sativum* radicles of various lengths (1 h D+MW/6 h D+MW = Desiccated for 1 and 6 h separately and then regrown in Mili-Q water, 1 h D+SA/6 h D+SA = Desiccated for 1 and 6 h separately and then regrown in SA). Bars represent Mean±SD of five independent observations. Values not related by same letter are significantly different at $p<0.05$ level

both the growth media, but its pace was quite high in case of MW. Conclusively, higher amounts of carbonyls (oxidized protein) were estimated in 15 mm radicles viz., 112.99 and 396.66 (in 1 and 6 h desiccated and MW regrown radicles) and 57.46 and 107.59 (1 and 6 h desiccated and SA regrown radicles) mM g^{-1} protein, respectively and their least (59.46, 157.03, 34.64 and 57.55 mM g^{-1} protein, respectively) in 1 mm radicles (Fig. 5b). In respect to growth media, amount of carbonyls formed in SA grown radicles of various lengths were considerably low than the MW grown radicles (Fig. 5b).

DNA content: Content of DNA was found to be increased with radicle elongation. Following desiccation

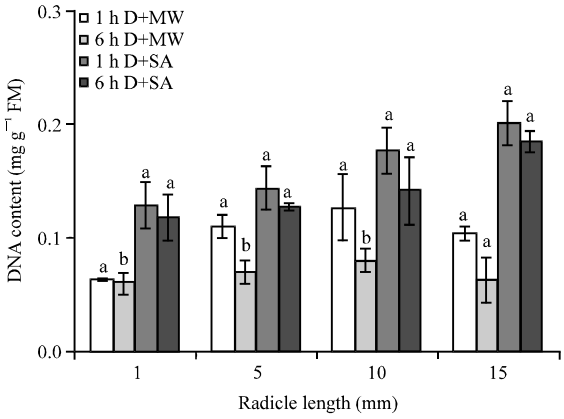

Fig. 6: Changes in DNA content of *Pisum sativum* radicles of various lengths subjected to desiccation-rehydration treatments (1 h D+MW/ 6 h D+MW = Desiccated for 1 and 6 h separately and then regrown in Mili-Q water, 1 h D+SA/6 h D+SA = Desiccated for 1 and 6 h separately and then regrown in SA). Bars represent means of five individual observations±SD. Values not related by same letter are significantly different at $p<0.05$ level

and re-growth in MW, a rise in DNA content was noticed between 1-10 mm radicles and was then declined (15 mm), whereas, it increases throughout in the SA raised radicles (Fig. 6). In regard to desiccation timing, lower amount of DNA was recorded in the 6 h desiccated radicles than its counterpart (1 h dried) (Fig. 6).

DNA oxidation and fragmentation: Following the methods of Langfinger and Von Sonntag (1985) and Ray *et al.* (1996), both oxidation and fragmentation of DNA was analyzed in pea radicles of varied lengths. But, even after applying possible alterations in the experimental protocols, we are failed to notice any significant change in above parameters, either in case of fresh or desiccated radicles of different lengths, regrown in both MW and SA.

DISCUSSION

In recent past successful attempts have been made to understand the mechanism of DT in an inducible system like germinated orthodox seeds. It was largely confirmed that induction of DT is possible in protruded radicles which are otherwise sensitive against dehydration (Buitink *et al.*, 2003; Faria *et al.*, 2005). Optimum radicle length beyond which seedlings of orthodox seeds lose DT varies species to species

(Faria *et al*., 2005). When *Brassica napus* seeds bearing 1.5 mm radicle was treated with ABA, tolerated drying as low as 0.34 g water g^{-1} dry mass without considerable loss of germinability (McKee and Finch-Savage, 1989). In this investigation, re-induction of DT was evaluated using germinated pea seeds as a model which exhibited 100% germination with a radicle of 1 mm length within 24 h and 15 mm radicle after 39 h of imbibition (Fig. 1). In the current investigation, 6 h desiccated radicle of 10 mm exhibited 20% survival percentage at 0.43 g water g^{-1}, whereas it was 10% only for 15 mm radicle with 0.46 g water g^{-1}, when subjected to MW (Fig. 2a-b and 3). Further, a significant promotion in the survival percentage, with delayed induction of DT in the growing Pea radicle was noticed, when dried (1 and 6 h) samples were re-grown in SA media (Fig. 2a, b and 3). Here, we have registered 70% survival for 6 h desiccated radicle of 10 mm length and 60% for 15 mm respectively when were allowed to re-grow on SA for next 24 h (Fig. 2a-b). We conclude that SA induced significant alleviation in both survival percentage and DT of pea radicles (Fig. 2a, b). Our results agrees with the observations of Buitink *et al.* (2003) and Faria *et al.* (2005) who have significantly promoted the rate of survival of germinated-desiccated *Arabidopsis thaliana* and *Medicago truncatula* seeds using ABA or PEG treatment respectively. They have further reported the successful re-induction of DT in these seeds/seedlings.

Authors have also showed that desiccation also imposes either permanent stoppage or lowering in the pace of re-growth of the seedling over suitable media (Buitink *et al*., 2003). However, rate of lowering in the radicle re-growth may depends basically on two factors; length of the radicle and degree of dehydration. In agreement, gathered data also revealed similar performance viz.: Speed of re-protrusion was anti-parallel to radicle length means highest increment was in the 1 mm radicle and lowest for 15 mm (Fig. 3). Additionally, pace of re-elongation declined in 6 h dried radicles compared to the 1 h, under both the regrowth media (Fig. 3).

Cellular membranes are one of the prime sites of desiccation injury and ROS were held solely responsible for it. Huang *et al.* (2012) has received a decline in the ROS with acquisition of DT and its increases with loss of DT. In accordance, we too have registered similar trend of ROS. In this investigation, a lower amount of ROS was seen in SA regrown radicles (Fig. 4a, b), exhibiting higher degree of DT (90-100%, Fig. 2b). Other side, ROS measured was statistically ($p<0.05$) higher in MW grown radicles exhibiting only 40-50% of DT (Fig. 2b). Considering accumulated data, we postulated that ROS content is intimately ($r = -0.90$, $p<0.05$) linked with acquisition of DT in MW radicles (Fig. 4a, b). In agreement, Hayat *et al*. (2010) has stated that addition of SA to desiccating organs of plant significantly reduces the ROS production ensuing enhanced growth and stress tolerance. Further, ROS was proven to play dual role in seed physiology, on one hand, it serves as a messenger in cellular signaling pathways at low concentrations and other side, as a cyto-toxic molecule if available in excess (Keshavkant *et al*., 2012). Looking to above contention, we can hypothesize that a fraction of the ROS produced during re-growth (particularly on SA media) of pea radicles, may possibly be playing a signaling role to complete the germination process.

It was recognized that storage protein accumulates in seeds at the time of their development and maturation (Olsen, 2001). Additionally, few of the stress related proteins synthesized/increased during maturation drying phase while others were induced at the time of desiccation. It means protein behave differently during protection against desiccation damage. Content of protein was evaluated to establish its association with DT of desiccated pea radicles (Fig. 5a). During evaluation, gradual reduction in protein level along with radicle elongation under both MW and SA rehydration was estimated (Fig. 5a). Although, the amount of protein measured was significantly ($p<0.05$) higher in the SA subjected radicles as compared to MW re-grown radicles of equal lengths (Fig. 5a). Our results are adhering with the observation of Anosheh *et al.* (2014) who have estimated higher contents of protein in SA supplemented *Hordeum vulgare* seeds compared to their control ones.

It has been proposed that carbonylation of storage protein would trigger their mobilization during germination by destabilizing the compact protein complexes thus increasing their sensitivity towards proteolysis (Parkhey *et al*., 2014). In the germinating white spruce seeds, traces of protein carbonylation was detected during early seedling growth later on, increased drastically with further elongation and rise in ROS (He and Kermode, 2010). In desiccated pea radicles of different lengths, we have estimated regular rise in protein oxidation reaction irrespective of re-growth media (Fig. 5b). More precisely, pace of carbonyl formation was initially slow but increased drastically in the radicles of 10-15 mm lengths probably due to increased ROS production (Fig. 5b). Other side, SA regrown seedlings exhibited limited accumulation of it (Fig. 5b). In parallel, in germinating megagametophyte of white spruce, protein carbonyl plays dual role; facilitates storage protein mobilization for germination, other side elicit signals for cell death if present in excess (He and Kermode, 2010).

Additionally, carbonylation has also been shown to have relevance in the completion of sunflower seed germination if exists within limits, otherwise plays deleterious functions (Oracz *et al*., 2007).

In seedlings, DNA content and degree of DT normally follow a high correlation (Boubriak *et al*., 2000), although the recommencement of DNA synthesis is unlikely to be the only effective agent in inducing the change from the tolerant to the sensitive state (Dasgupta *et al*., 1982). As DNA replication is normally a late event during seed germination, few other processes may be more closely linked to the loss of DT, with DNA content playing only an additive role in the increasing stress sensitivity upon germination (Boubriak *et al*., 2000). In PEG treated *Medicago truncatula* seedlings, the greatest drop in DT occurred with increasing 4C DNA content and a second cause for drop of this DT observed was increase in the length of protruding radicle (Faria *et al*., 2005). Here, content of DNA remain unchanged but a great number of cells had entered the M phase of the cell cycle. In support, it was largely reported that dividing cells are less tolerant to desiccation than those that are elongating (Dasgupta *et al*., 1982). In the current study, we have measured regular increase in DNA content between 1 to 10 mm radicle lengths, irrespective of desiccation timing and re-growth medium (Fig. 6). But, we have not attempted to confirm the state of DNA, whether they may be at 2C stage or 4C. After concerning literature, we can only assume that MW grown radicles must be in their 4C stage consequently conferring desiccation sensitivity (Fig. 6). Salicylic Acid (SA) was widely shown to activate all sort of protective molecules and systemic acquired resistance in plants against variety of stresses. It is also reported to remove hydroxyl radicals completely from stressed tissues, which are dedicatedly prone towards DNA damage (Anosheh *et al*., 2014). Keeping this, it can be assumed that SA also takes part in protection/maintenance of DNA in stressed cells by one or other mechanisms.

In general, desiccation of intolerant or germinated orthodox seeds imposes fractionation in their DNA (Boubriak *et al*., 2000). After working with *Medicago truncatula* seedlings, Faria *et al*. (2005) has demonstrated that drying of seedling (2 mm) induces degradation of nuclear DNA. In contrast, in DT seed damaged DNA was successfully repaired with reintroduction of water (Boubriak *et al*., 2000). However, DNA laddering is an indicator of end point of the apoptotic process and can not be reversed back (Boubriak *et al*., 2000). In the current demonstration, we were unsuccessful to detect traces of DNA oxidation/ fragmentation, in pea radicles. Supportive to ours, it has been argued that mild stress can initiate synthesis of sugars and LEA proteins, serving as protective agents, thereby stabilizing cellular components as well as structures (Buitink *et al*., 2003). It can thus be speculated that rehydration induces synthesis of nuclear proteins, bearing DNA protective function. It is also thought that desiccation induced nuclear protein; QP47, isolated from pea seed protects DNA during desiccation injury (Chiatante *et al*., 1995). Besides the synthesis of protectants, water loss may also cause reversible conformational changes in the DNA disturbing the recognition of specific base sequence domains by enzymes (Osborne *et al*., 2002) thereby hindering the action of the nucleases although this is yet to be investigated in plant cells.

CONCLUSION

In conclusion, desiccation tolerant organisms must rely on one or both of the following mechanisms: (1) Avoidance of the accumulation of dehydration mediated damages and (2) Activation of repair mechanism upon rehydration. Current investigation exhibited that both mechanisms were distinctly applied by radicles in which DT was reestablished with SA supplementation. In Pea radicles, ROS and protein content was correlated well with loss of DT. Additionally, nuclear DNA was kept intact during dehydration.

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