

Full Length Research Paper

The impact of salinity stress on morphological and anatomical aspect of water hyssop *Bacopa monnieri* (L.) Wettst grown *in vitro*

Abdulminam Hussien Ali Almusawi^{1*}, Haleemah Jabbar Al-Aradi² and Kadhim Jasim Hammadi³

¹Cell Research and Biotechnology Unit, College of Science, Basrah University, Basra, Iraq.

²Marine Science Centre, Basrah University, Basrah, Iraq.

³Biology Department, College of Education, Basrah University, Basrah, Iraq.

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This study was conducted at Marine Science Center, Basrah University, Basrah, Iraq during the period of 2015 to 2016. In the present work, we used NaCl and polyethylene glycol (PEG) to induced abiotic stress *in vitro*. Results show that high shoot numbers (24) were produced on MS (solid) medium enriched with 0.8 g/L NaCl in addition to cytokinins while, inclusion of drought agent PEG to proliferation medium (solid and liquid) produced a minimum shoots of 5 and 11 respectively. Root growth and development were also investigated in this study. It was found that high root development was observed on MS (solid or liquid) medium free of hormone (control) and MS (solid or liquid) medium containing 0.8 g/L NaCl 26.67, 25, 21.67 and 18 respectively whereas, less root development was obtained on MS medium (solid or liquid) enriched with NaCl and 26 g/L PEG. In this study it was found that inclusion of the amino acid proline to the stressed media did not improve the growth of shoots or roots. Histological analysis of multiplied shoots under abiotic stress agents showed accumulation of insoluble starch granules in the parenchyma cells of the cortex layer and this may be a part of protective tolerate mechanism used by medicinal hyssop (*Bacopa monnieri*) against abiotic stress.

Key words: *Bacopa monnieri*, medicinal hyssop, Brbin, micropropagation, salt stress, polyethylene glycol (PEG), proline.

INTRODUCTION

Bacopa monnieri (L.) Wettst a water hyssop, known as Brahmi, or Brbin belonging to the family Plantaginaceae is widely distributed in warmer parts of Asia, Australia, and America. It is an important medicinal plant used in the

Ayurvedic system for centuries. This creeping herb with a light purple flower is used for various ailment but is best known as a brain tonic, a nerve tonic for enhancing memory, improves intellectual and cognitive functions,

*Corresponding author. E-mail: abdulminamhussienali@gmail.com.

anti-inflammatory, analgesic, antipyretic, sedative and antiepileptic agent. In India and Pakistan this plant has been used as a cardiac tonic, digestive aid and improves the respiratory system (Bammidi et al., 2011; Charoenphon et al., 2016). It also used in medical therapy for insomnia, asthma, hoarseness, snake bite, rheumatism, leprosy, eczema, water retention, blood cleaning and insanity (Banerjee and Modi, 2010).

Environmental stress and in particular salinity is one of the important factor that limits the distribution, crop productivity, morphogenesis and the formation of secondary metabolites of the glycophytic plant (Liu and Cheng, 2008; Charu et al., 2015; Vibhuti et al., 2015; Awasthi et al., 2016). However, plants have developed complex mechanisms to adapt to osmotic, ionic and oxidative stresses induced by salt stress; for instance, the accumulation of proline (Debnath, 2008; Bargali and Bargali, 2016). Tissue culture is used to evaluate the impact of abiotic stress on cell metabolism morphologically, anatomically and physiologically; it also opens a new path for the production of secondary metabolites (Debnath, 2008; Naik and Al-Khayri, 2015). Tissue culture is a novel technology used to investigate plants' tolerance to different abiotic stresses factors. So, the present study aimed to investigate the effect of salinity (NaCl) and drought agent *polyethyleneglycol* (PEG) on morphogenetic potential and histological aspect of *in vitro* grown shoots of *Bacopa monnieri*. (L.) Wettst.

MATERIALS AND METHODS

Source of the plant materials and surface sterilization

This project was conducted at Marine Science Center, Basrah University, Basrah, Iraq during the period of 2015 to 2016. A plant was collected from different parts of Shatt- Al-Arab coast. Young and healthy shoots were selected as a material for *in vitro* culture use. Shoots were treated with 10% (v/v) liquid detergent solution for 5 to 10 min, followed by rinsing under running tap water for 15 to 20 min. Shoot tips of 1 to 2 cm were detached from the young shoots and immersed directly in antioxidant solution consisting of 100 mg/L ascorbic acid and 150 mg/L citric acid until the time of sterilization. The clean shoot tip explants were treated with 20% (v/v) bleach solution containing two drops of Tween-20 per 100 ml as a surfactant for 10 min under aseptic conditions (Laminar flow air cabinet). Shoots were rinsed three times with sterile distilled water to remove the traces of bleach solution.

In vitro culture establishment

Surface disinfectant apical buds were inoculated onto Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) having 3% sucrose and supplemented with 0.5 mg/L benzyl adenine (BA) + 0.5 mg/L kinetin (AL-Arabi et al., 2016), and MS vitamins. pH of the medium was adjusted to 5.8. Then, 0.6% agar was added. The medium was then autoclaved at 121°C for 20 min. *In vitro* generated healthy shoots were maintained by regular subculturing of propagules of four shoots each after every 8 weeks of culture. Shoots explants obtained from the *in vitro* cultures were used as inoculum and subjected to different abiotic stress treatments.

In vitro abiotic stress treatments

To determine the abiotic stress tolerance of medicinal plant *B. monnieri* by examining morphological and histological changes following water stress, the multiplied shoots were transferred to same proliferating MS (liquid and solid) medium containing different abiotic stress as follow: NaCl (0.8 g/L) and NaCl 0.8 g/L with 26 g/L polyethyleneglycol or 50 mg/L proline. Cultures were grown in a growth chamber at $27 \pm 2^\circ\text{C}$ with photoperiod of 16 h day/dark of $10 \mu\text{mol m}^{-2}\text{s}^{-1}$.

To study the effect of abiotic stress on root development, multiplied shoot mass was transferred to hormone free MS (solid and liquid) medium fortified with different abiotic stress as follow: NaCl (0.8 g/L) and NaCl 0.8 g/L with 26 g/L polyethyleneglycol or 50 mg/L proline medium. Cultures were grown in a growth chamber at $27 \pm 2^\circ\text{C}$ with photoperiod 16 h day/dark of $10 \mu\text{mol m}^{-2}\text{s}^{-1}$.

Regeneration potential development

Shoots and plantlets from all stressed media were transferred to medium free of abiotic stress agents for shoot and root regeneration. Cultures were maintained at temperature $27 \pm 2^\circ\text{C}$ and 16 h photoperiod of $10 \mu\text{mol m}^{-2}\text{s}^{-1}$.

Data recording and statistical analysis

Experiments for shoots multiplication and rooting stress under normal and abiotic stress condition *in vitro* were repeated thrice. For each treatment, five tests tubes or jars were used. The data with respect to shoot and root numbers were recorded for each treatment after one and two months. Same date recording used for shoot and root regeneration after stress removed. All data were subjected to Analysis of Variance (ANOVA) for a completely randomized design (CRD). The differences among the treatment means were tested by Duncan's new multiple range test (DMRT) (Gomez and Gomez, 1984).

Microscopic study

A comparative histological study was conducted on the shoot for different treatments mentioned above in addition to control. Specimens were killed and fixed in (formalin: acetic acid: alcohol 90:5:5) (FAA) for 24 h, then dehydrated in ethanol series using 50, 70, 80, 90% and absolute 100% concentrations (Johanson, 1940). Specimens were then embedded in paraffin (solidification point about 57 to 60°C) and sectioned to thickness 10-micron sections. The sections were double-stained with safranin and fast green. Sections cleared with xylene and mounted in Canada balsam (Yilun et al., 1992). Slides were microscopy examined.

RESULTS AND DISCUSSION

Bud induction and shoot proliferation

It was observed that the addition of cytokinins to MS medium was essential for bud development and proliferation. Apical bud grown on hormone free medium showed less response and bud later died (data not shown in this paper). Proliferated shoots were transferred to same fresh medium after every eight weeks. Similar results were obtained by Binita et al. (2005), Sharma et al. (2010), Sundriyal et al. (2013) and Jain et al. (2014).

Table 1. Typical response for shoot numbers of *Bacopa monnieri* on liquid and solid medium, supplemented with 0.5 mg/L BA + 0.5 mg/L free of salinity and drought agents and subjected to salinity (NaCl), proline and drought (PEG) stress after one and two months.

S/N	Treatments	Rate of shoots number			Rate of roots numbers		
		After one Month	After two months	Regeneration potential (re-growth after stress relieved)	After one Month	After two months	Regeneration potential (re-growth after stress relieved)
1	MS solid medium (control)	25 ^b	28.7 ^b	29.33 ^a	18 ^a	26.67 ^a	21 ^a
2	MS liquid medium (control)	33.33 ^a	42.3 ^a	24 ^{ab}	24 ^a	25 ^{ab}	20.7 ^a
3	MS + 0.8 g/L NaCl (solid)	5 ^c	24 ^b	22 ^{bc}	5 ^b	21.67 ^{ab}	19.3 ^a
4	MS + 0.8 g/L NaCl (liquid)	6 ^c	15.7 ^{bcd}	19.33 ^{bcd}	5 ^b	18 ^{bc}	19 ^a
5	MS + 0.8 g/L NaCl + 50 mg/L proline (solid)	3.33 ^c	13.7 ^{cd}	15.33 ^{cde}	4.67 ^b	12.67 ^{cd}	16.3 ^a
6	MS + 0.8 g/L NaCl + 50 mg/L proline (liquid)	7.33 ^c	12 ^{cd}	12.33 ^{de}	5.67 ^b	11.67 ^{cd}	14 ^a
7	MS + 0.8 g/L NaCl + 26 mg/L polyethelenglygol(solid)	3.33 ^c	5 ^d	12 ^{de}	6 ^b	10 ^{cd}	12.3 ^a
8	MS + 0.8 g/L NaCl+ 26 mg/L polyethelenglygol (liquid)	7.67 ^c	11 ^{cd}	9.33 ^e	7.33 ^b	7.67 ^d	11 ^a
L.S.D.		6.96	13.07	7.309	8.443	8.406	11.29

Table 1 shows that the highest shoot numbers were obtained on liquid MS medium supplemented with 0.5 mg/L BA + 0.5 mg/L kinetin and free of salinity and drought agents after one and two months; 33.33 and 42.2 respectively (Figure 1A and B). These results are in line with those of Tiwari et al. (2000), who proposed an efficient and rapid method for *B. monnieri* by using a liquid medium. Moreover, the MS medium fortified with 0.5 mg/L BA + 0.5 mg/L kinetin liquid or solid medium showed no rosette clump; so it has been used for this study. *In vitro* shoots explants from control cultures were used as inoculums and subjected to salinity (NaCl) and drought (PEG) stress.

***In vitro* stress treatments**

Concerning the number of shoots, the results illustrated in Table 1 shows that maximum shoot numbers rate (24 culture) were produced on MS (solid) medium supplemented with 0.8 g/L NaCl after two months. The most harmful effects of multiplied shoots were recorded on MS solid medium containing 0.8 g/L NaCl +26 g/L PEG, where, the less average shoot numbers (5 shoot/culture) were recorded in this medium after two months respectively (Table 1). Results also recorded some yellowing on multiplied shoots grown on MS media containing stress agents and no necrosis was observed (Figure 2A and C). But, in spite of a long-term exposure to salinity and drought stress for two months, shoot

cultures were still viable. These findings indicated that *B. monnieri* can tolerate both NaCl and other water stress agents (Figure 2A to C). Table 1 also shows that cultures resumed their growth when stress relieved, and the shoots number increased distinctly compared with cultures subjected to stress. An identical observation was reported in same species *B. monnieri*, where plants are able to tolerate water stress for 45 days (Debnath, 2008).

Growth reduction in tissues grown in stressful media *in vitro* is the usual phenomenon, but stress metabolism resistance is really interrupted (Misra et al., 1997, Debnath, 2008). So, the response of shoots multiplication or roots formation in salinity and drought stress were different (Table 1).

NaCl stress involves both osmotic and ionic stress agents but the PEG is a high molecular weight osmotic agents. However, salinity and drought-induced production of active oxygen species (AOS) often cause oxidative stress (Sgherri et al., 2000; Khan and Panda, 2008). However, plants have a specific protective mechanism in various degree for defending themselves against activated oxygen (Sudhakar et al., 2001). In the present study, a significant statistically difference between shoots numbers of control and others of water stress treatments were recorded (Table 1 and Figure 2C) but, all the stressed plants showed the non-significant decrease in shoot numbers after one and two months. In the presence of both salinity and drought agent (PEG) in a liquid medium, a less shoots number was obtained; 3 and 5 shoots after 1 to 2 months because the (PEG) restrict

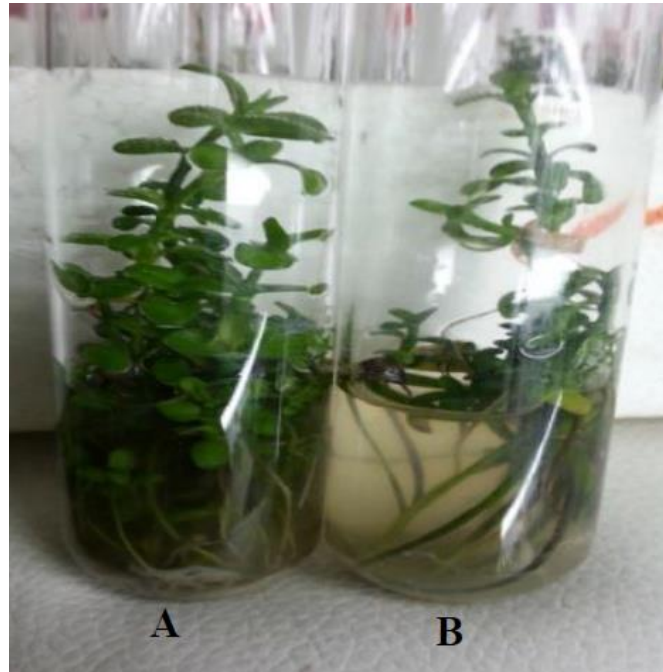


Figure 1. Initiation of shoots from apical bud on MS medium containing 0.5 mg/L BA + 0.5 mg/L kinetin. **A**, Shoot proliferation on liquid medium. **B**, solid medium.

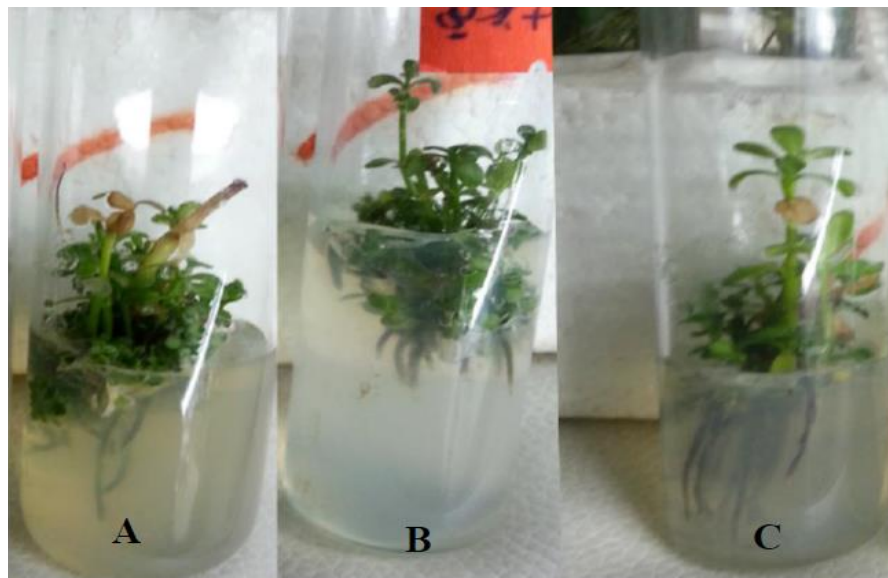


Figure 2. Shoot proliferation on MS solid medium containing abiotic stress agents. **A**, 0.8 g/L NaCl. **B**, 0.8 g/L NaCl + proline. **C**, 0.8 g/L NaCl + PEG.

the water translocation through plant inhibiting leaf expansion and plant growth (Yeo and Flowers, 1994). This restriction may affect shoot growth and proliferation by reducing the media uptake by shoot explants.

Concerning root growth and development, the impact of different abiotic stress represented in Table 1 shows that

the best root numbers were obtained on MS solid and liquid medium free of hormone and abiotic stress agents after one and two months were 18, 24, 26.67 and 25, respectively. The result also revealed that addition of salinity agent (NaCl, 0.8 g/L) to the medium did not decrease the root numbers. Instead, a high root number

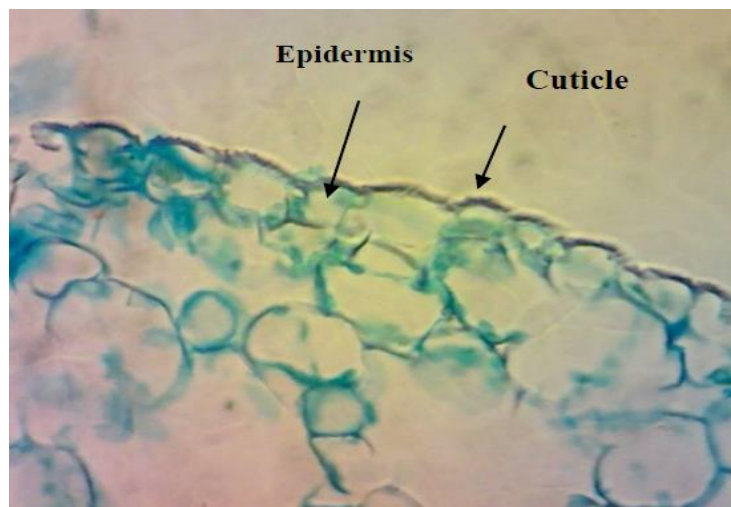


Figure 3. Cross section of stem: epidermis cells of stem cultured on MS medium free of abiotic stress and MS medium enriched with salinity, proline (anti abiotic stress) and drought (PEG) stress. No variation in cuticle thickness has been detected (200 times magnification).

(21.67 and 18) was recorded from shoots grown on MS (solid and liquid) medium containing salinity agent (NaCl, 0.8 g/L) after two months. However, on the rooting medium, the multiplied shoot on various stress media showed no significant increase in shoot growth after 1 to 2 months, and instead of that, it morphologically shifted from shoot proliferation to root formation (Table 1). This result is in accordance with that of Debnath (2008) who observed same phenomena on the *B. monnieri* hyssop cultured *in vitro* under stress agents while, other researchers (Ehsanpour and Amini, 2003; Misra et al., 1990) suggested that increasing the root length and branching is one way of tolerance mechanism against drought and salinity stress.

Results also revealed that combined PEG to the culture media (solid and liquid) decreased the roots numbers to 10 and 7.67 respectively. This finding is in agreement with those of Said et al. (2015) who found that root growth parameters markedly decreased in Banana plantlet cultured *in vitro* when PEG was included in the media. Results also shows that adding the proline to the MS medium containing salinity agent alone or in combination with drought agent (PEG) did not improve the shoot multiplication or root development (Table 1).

After stress, relieved numbers of roots showed insignificance difference between control and other stress treatments (Table 1).

Histological study

Anatomical studies of plants under stress are very important because they show adaptive features of plants under these conditions (Younis et al., 2013). Histological study of *B. monnieri* stem subjected to various stress

treatments was conducted to describe its response to different abiotic stress *in vitro*. It was noticed that the epidermis of stems cultured in various salinity and drought stress agents, as well as control media, consist of one layer with thin-walled cells, no variation in cuticle thickness has been recognized (Figure 3). Stems grown in different abiotic stress agents' media showed high insoluble starch granules accumulation, in parenchyma cells of the cortex (parenchyma) (Figure 4B, C and D); whereas, no starch granules were recognized in stem grown in control medium (stress-free medium) (Figure 4A). The vascular area (phloem and xylem) of stems subjected to various abiotic stresses is wide, distinct and contained high numbers of vascular bundles in compared with control (Figure 5B). For the control, the vascular area is very small; fewer numbers of mature vascular bundles was detected (Figure 5A). So, anatomical results indicated that starch accumulation, as well as wide vascular area, are a possible productive tolerate mechanism use for salinity or drought stress in *glycophytic hyssop B. monnieri*. However, no phenolics or damaged parenchyma cells have been recognized in all sections stems subjected to drought or salinity stress treatments. These findings are paralleled to those of Theerawitaya et al. (2015) study, who demonstrated that the accumulation of soluble and insoluble starch granules was detected in rice seedling cultivars IR29 and Pokkalid, depending on the salts stress exposure times and plant organs.

Conclusion

Salinity and drought stress are the most important environmental factors that restrict glycophytic plant

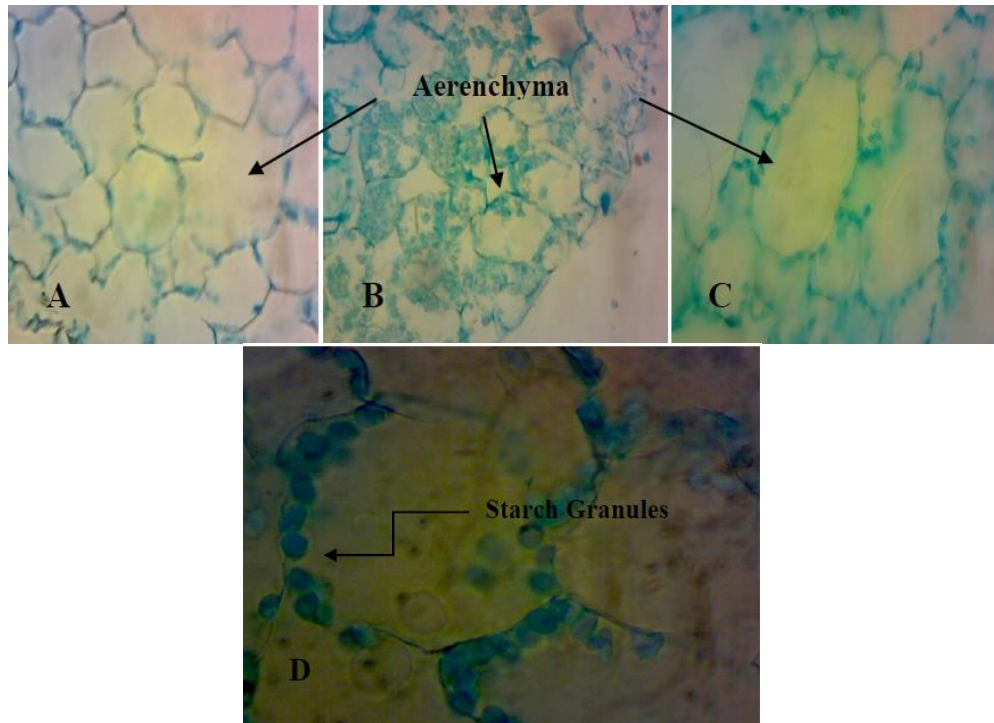


Figure 4. Cross section of stem. **(A)** Parenchyma cells of stem cultured in control medium (free of a biotic agents), no starch granules accumulation has been recognized (100 times magnification). **(B)** Parenchyma cells of stem cortex grown on MS medium enriched with salinity, proline (anti a biotic stress) and drought (PEG) stress, agents, parenchyma cells seems filled with insoluble starch granules (100 times magnification). **(C)** Parenchyma cells of stressed stem (200 times magnification). **(D)** Parenchyma cells of stressed stem under high power of magnification (400 times).

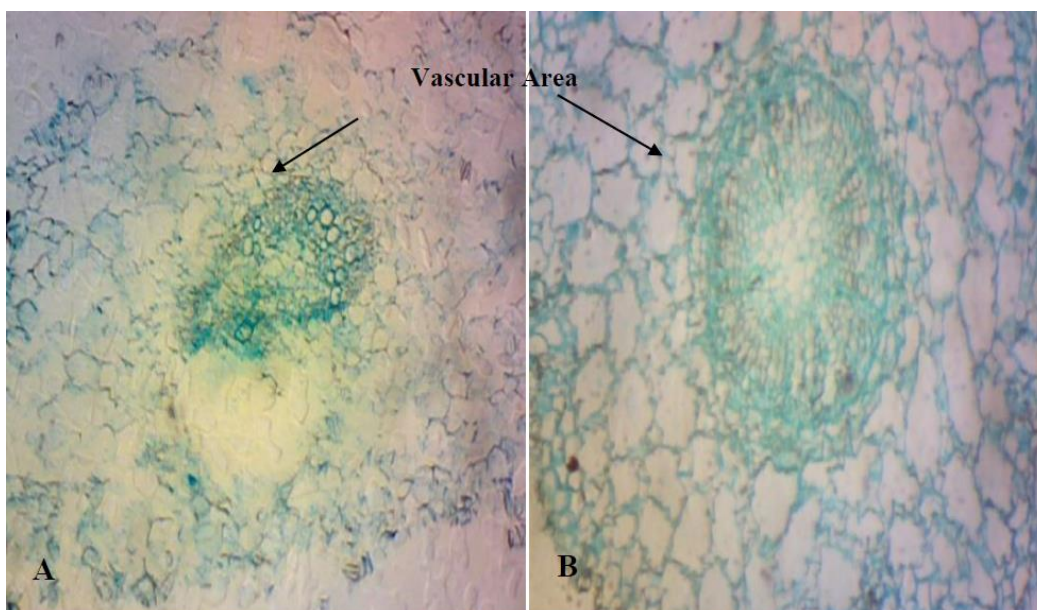


Figure 5. Cross section of stem: vascular of stem cultured in MS medium free of abiotic stress (A) and MS medium enriched with salinity, proline (anti a biotic stress) and drought (PEG) stress (B). (A) For stem grown in control medium, vascular area is very small with less numbers of mature vascular bundles. (B) For stems cultured under stress conditions, vascular area were more distanced and matured (40 times magnification).

growth. In this study we conclude that the deleterious effect on shoot multiplication and root development comes when PEG-containing medium was used. No adverse effects were recognized on shoot or plantlets grown on MS medium containing 0.8 g/L NaCl. Histological study revealed that the shoots grown under abiotic stress had a mature vascular area compared with the control. These preliminary results open the door to use a low salinity agent (0.8 g/L NaCl) instead of PEG and paclobutrazole to promote *in vitro* hardening.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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