Section A-Research paper

Screening of pea (*Pisum sativum* L.) accessions for drought stress tolerance based on leaf disc assay and stomata density

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Abstract

Pea is one of the most significant protein-rich pulse crops grown in various regions of the world; thus, it is an important member of the agroecological cropping system. Several morphological and physiological parameters, such as seed germination, plant height, root length and weight, total biomass, flower development, photosynthesis rate, chlorophyll content, and antioxidant activity, directly depend on the potential of particular genotypes/accessions. The objective of the present study was to identify drought-tolerant pea accession based on leaf disc and stomata density assay. Leaf disc and stomata density assays have some advantages over whole-plant assays because they require less sample preparation time without harvesting of plants and allow a higher number of replicates. Leaf samples of fifteen pea accessions and two commercial varieties were used for leaf-disc and stomata density assay. Drought condition was generated by mannitol (100mM and 150mM) in the laboratory to check stress-tolerant pea accession. After one week, it was observed that two accessions ARG6 and ARG10 were stress-tolerant, as the colour of the disc remained green in 150mM and 200mM mannitol in compared to other pea accessions. Furthermore, low stomatal density was observed in ARG6 and ARG10 pea accession. Maximum chlorophyll content was observed in two pea accessions namely ARG6, and ARG10 and one

commercial variety i.e., PS10 in comparison to their control. Similarly, lower stomata density was observed in ARG6, ARG10 and PS10. These results determine the magnitude of the effects of drought stress under the test conditions. Based on the leaf disc, stomatal density, and chlorophyll content, two pea accession, ARG6 and ARG10 and variety PS10 were screened and selected as tolerant plants.

Keywords: Pea (*Pisum sativum*), Leaf-disc assay, Stomata density and chlorophyll content

1. Introduction

Drought stress negatively affects plant growth and yield by interrupting physio-biochemical processes. Various attributes, such as structural changes, water use efficiency, transpiration, osmotic adjustment, osmolyte accumulation, enhanced antioxidant enzymes, and free radical quenching systems are some of the important and effective strategies that plants may use to adapt to abiotic stress (Tuteja et al., 2011). The effect of drought stress on plants is clearly visible in its morphological aspects, such as the height, root length, leaf number and size, biomass and in biomass portioning.

Drought occurs when rain precipitation is significantly below the normal recorded level, and is a naturally occurring phenomenon that causes serious hydrological imbalances (According to the United Nations Convention to combat desertification UNCCD). Drought is also known as a water deficit condition. Many literature reviews have discussed that global warming is one of the reasons why drought occurs frequently in some places. Drought stress disrupts the membrane structure and osmotic equilibrium of the cell. At the time of drought stress, the cell membrane loses its selectivity and integrity and loses its enzymatic activity (Seleiman et al., 2021). This causes a reduction in water in the lipid bilayer, which ultimately results in protein-membrane

displacement. The plant closes its stomata, activates respiration, restrains cell growth, and starts shedding older leaves these processes are done to avoid drought stress at the physiological level (Shinozaki et al., 2007).

Pisum sativum L. is a herbaceous plant, also known as a garden pea. It is one of the most cultivated legumes around the world followed by soybeans, groundnuts, and beans (Mishra et al. 2012). Peas are also known as green manure, which is cultivated so that the soil can capture nitrogen from the roots of legumes. Most legumes have root nodes in their root fibres, which contain symbiotic bacteria that help in nitrogen fixation. These root nodes are the main reason legumes are rich in protein, as they conserve nitrogen (Postgate, 1998; Smil, 2000).

Pea plant is brutally affected by drought stress (Murray et al., 1981). Under drought conditions, pea plants move towards early fruiting, less total biomass, and low transcription, which causes stomatal closure. The present study was based on the screening of drought-tolerant and drought-sensitive pea plants with the help of leaf disc assay, stomatal density, and chlorophyll content in the leaves of plants. These assays do not require scarification of the plant, and one or two-leaf samples were used for this study.

2. Materials and methods

Plant material- Fifteen accessions of *Pisum sativum* (pea) were procured from IARI Delhi, and two commercial pea varieties (PS-10 and P-3) seeds were purchased from a local market (Gwalior) in 2019. The seeds were germinated in control conditions in the plant tissue laboratory at optimum temperature (18-20°C temperature), 16 hours light and 8 hours dark conditions till 8 leaf-stage. After thirty days of germination, they were transferred to the pot in a greenhouse.

Sterilization and germination of seeds-

Accessions of pea plants were germinated under sterilized and laboratory conditions. The seeds were sterilized with detergent for five minutes, washed with distilled water and 70% ethanol for two minutes, dipped in 0.05% $HgCl_2$ for three minutes and at last, $HgCl_2$ was washed out by three times with distilled water, and placed on MS media inside the phytazar in the plant chamber (Ramakrishna et al., 1991).

Leaf disc assay:

The leaf disc assay was performed for the screening of drought-resistant peas. In this method, leaf samples were collected from every plant in triplicates and cut into a disc shape that represents the physiological response of plants to internal and external stimuli. The protocol for the leaf disc assay was modified (Chiu et al., 2021). Stress on the leaf discs was provided by 100mM mannitol and 150mM mannitol. The leaf pea plants from all the accessions were collected and cut into circular discs. Three petri-plate having water (Control), 100mM mannitol and 150mM mannitol respectively was prepared. Ten leaf discs were transferred into the Petri-plates. The Petri plates were placed in **a** plant tissue laboratory at optimum temperature (18-20°C temperature) and 16 hours light, 8 hours dark conditions. The leaf samples were observed every 3^{rd} day.

Chlorophyll content

DMSO solvent was used to carry out the chlorophyll extraction from the leaves. 25mg of leaves from each plant accession (Controlled and stressed) were chopped into small square pieces and placed in test tubes containing 5ml of DMSO. The tubes were placed in the dark for 6 to 8 hours (Manolopoulou et al., 2016).

Chlorophyll concentration (a, b, and, total pigments) was determined by the following formula.

Chlorophyll a (mg/g F.W) = 14.85 A665 -5.14 A648

Chlorophyll b (mg/g F.W) = 25.48 A665 – 7.36 A648

Total chlorophyll (mg/g F.W) = 7.49 A665 + 20.34 A648

Where, A665 = absorption value at 665 nm, A648= absorption value at 648 nm.

Stomata density

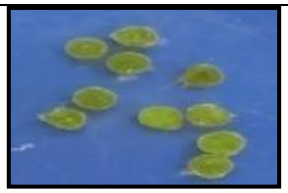
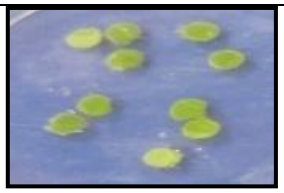
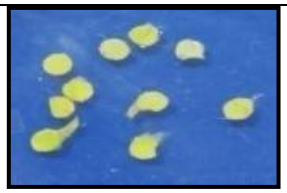
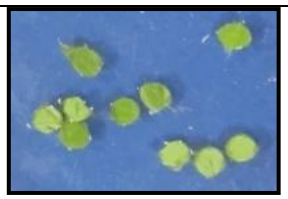
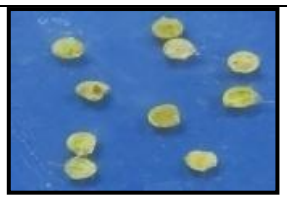
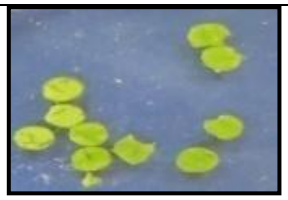
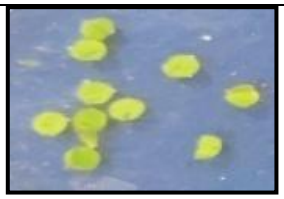
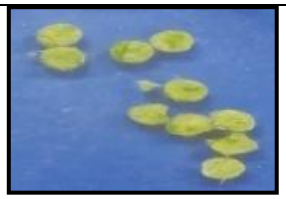
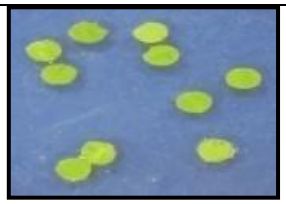
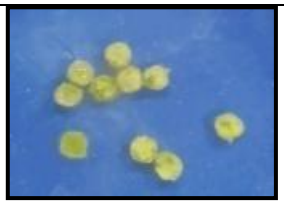
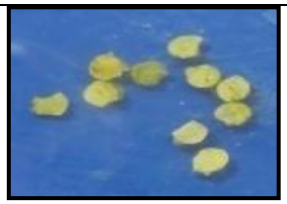
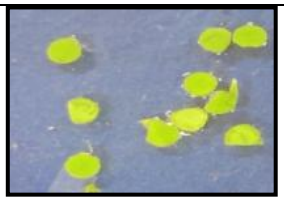
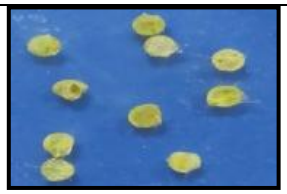
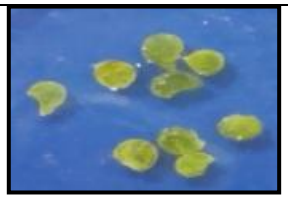
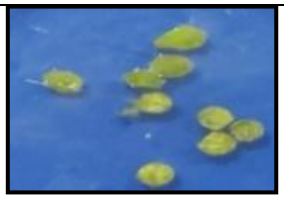
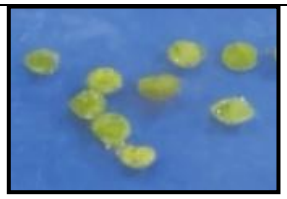
Stomatal density was measured by the clear nail varnish method. An epidermal impression was prepared by coating the nail varnish on the leaf surface. The dried layer of the nail varnish was peeled off using clear tape and put it onto a slide. The slide was observed at X400 magnification (Zhu et al. 2018).

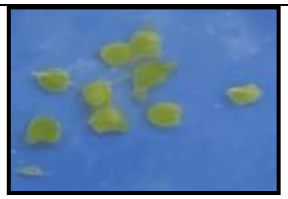
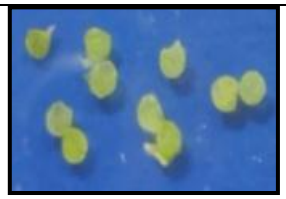
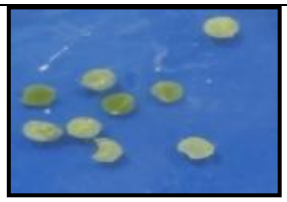
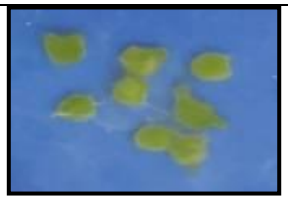
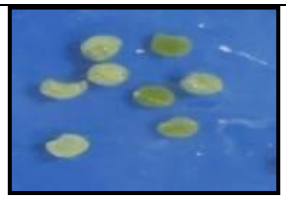
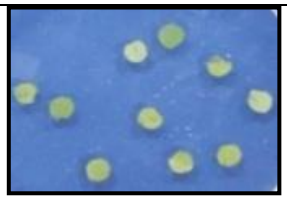
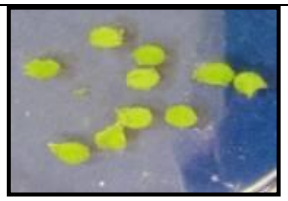
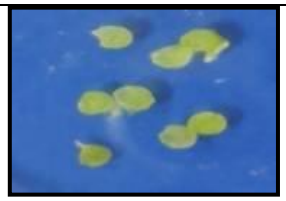
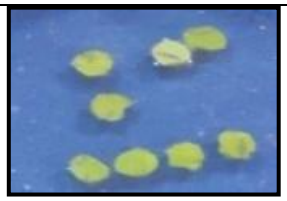
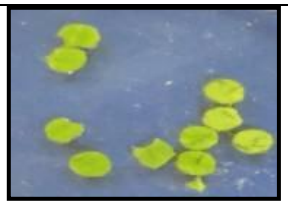
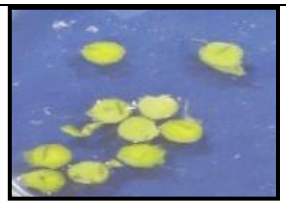
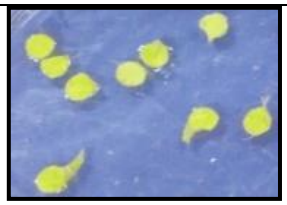
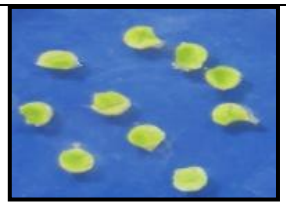
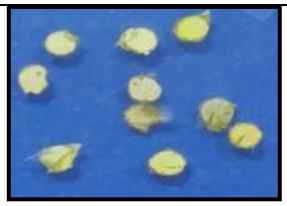
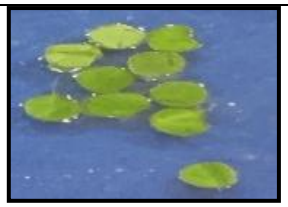
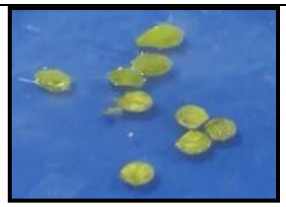
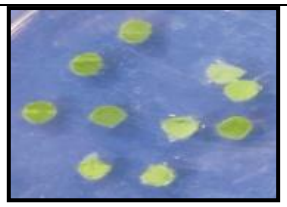
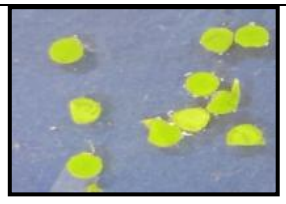
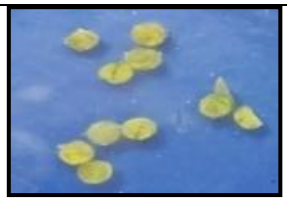
3. Results

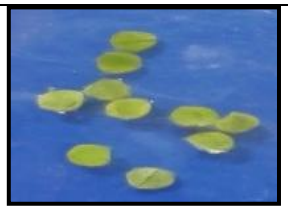
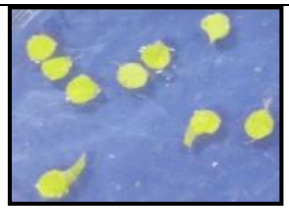
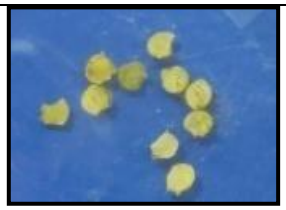
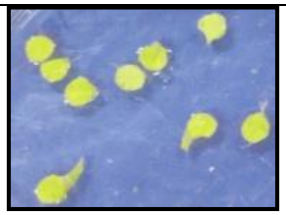
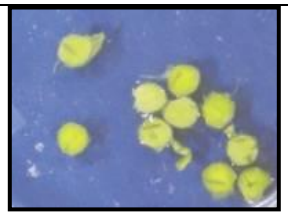
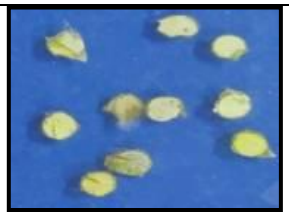
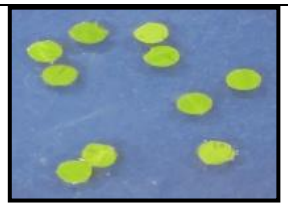
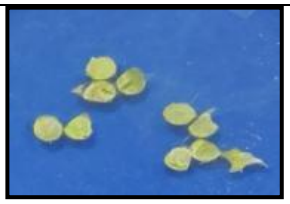
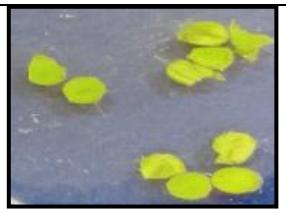
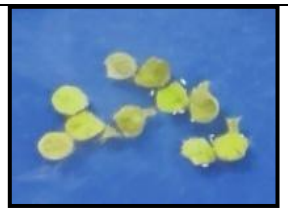
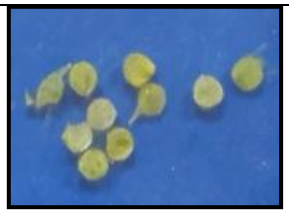
Leaf disc assay

After 30 days of germination, similar plants (Height, leaf number) were selected for stress (drought) study. Leaf disc assay was conducted to screen the resistant and sensitive plants under drought conditions without harvesting the entire plant. The leaf disc was conducted under controlled conditions. After incubation (7 days) of the leaf disc under stress conditions, low discolouration was observed in the control plant leaf. However, a progressive decrease was observed as the drought stress levels increased. Out of 17 (15 Pea accession and 02 commercial variety) pea plants in control condition maximum pigment was observed in mostly all plants. While under treated conditions the maximum pigment was observed in ARG6 and ARG31 (Table- 1).

Table 1: Leaf disc assay of pea accessions through 100mM and 150mM mannitol solution

S.No.	Plant	Control	100mM Mannitol	150mM Mannitol
1	ARG3			
2	ARG5			
3	ARG6			
4	ARG9			
5	ARG10			
6	ARG13			

7	**ARG20**			
8	**ARG24**			
9	**ARG27**			
10	**ARG31**			
11	**ARG33**			
12	**ARG34**			
13	**ARG35**			

14	**ARG37**			
15	**ARG39**			
16	**PS-10**			
17	**P-3**			

Pigment analysis

a) **Chlorophyll 'a'**

Chlorophyll content was also observed in control and treated plants. Based on leaf disc assay, six pea plants were selected for further Chlorophyll a & b study. Chlorophyll 'a' content was observed higher in the control of each six accession however, a progressive decrease was observed as the drought stress levels increased. The study revealed that the average chlorophyll 'a' content under the control condition was 9.4±2 whereas the average chlorophyll 'a' content under the stress condition was 7.8±1.5. Out of 6 selected pea plants in control, the maximum chlorophyll 'a' content was observed at 11.9 ±0.4 (in P-3) whereas the minimum chlorophyll 'a'

content was observed 6.1±0.8 (in ARG24). While under treated conditions the maximum chlorophyll 'a' content was observed in ARG6 and PS-10 in comparison to their control (Figure-1).

b) **Chlorophyll 'b'**

Chlorophyll 'b' content was observed higher in each control plant however, a progressive decrease was observed as the drought stress levels increased. The study revealed that the average chlorophyll 'b' content under the control condition was 16.3±2.3 whereas the average chlorophyll 'b' content under the stress condition was 13.6 ±3.3. Maximum chlorophyll 'b' content (19.8 ±0.3) was observed in the control ARG10 plant whereas the minimum chlorophyll 'b' content (12.2 ±0.3) was observed in ARG24. While under treated conditions the maximum chlorophyll 'b' content was observed in ARG6, PS-10 and moderate in ARG10 compared to their control (Figure-1).

c) **Total Chlorophyll**

Total chlorophyll content was observed higher in the control however a progressive decrease was also recorded as the drought stress levels increased. The study revealed that the average total chlorophyll content under the control condition was 10.8±1.6 whereas the average total chlorophyll content under the stress condition was 8.4 ±2.4. Out of 6 selected pea plants in control, the maximum total chlorophyll content (13.2 ±0.2) was observed in ARG10, whereas the minimum total chlorophyll content (9.5±0.3) was observed in ARG24. While under treated conditions the maximum total chlorophyll content was observed in ARG6, PS10 and moderate in ARG1O in comparison to their control (Figure-1).

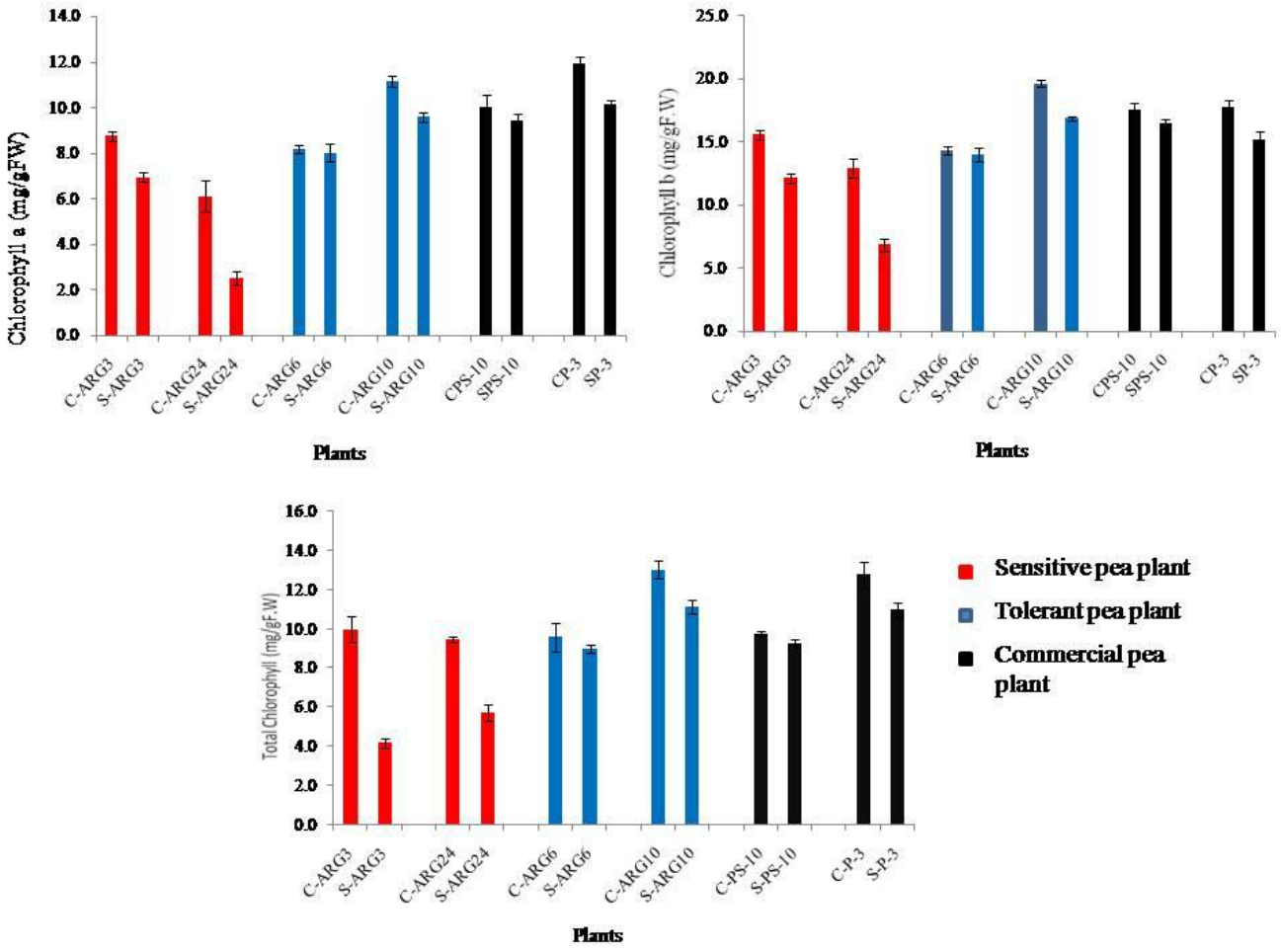


Figure 1- Chlorophyll 'a', 'b' and Total Chlorophyll of Drought Stress tolerant and sensitive accessions

Stomata density

The stomata of pea plants were studied under a 40X microscope. In all the pea plants, the stomata density studied was higher in the control. However, a progressive decrease was observed as the drought stress levels increased. The study revealed that the average open stomata density under the control condition was 27.4 +3 whereas the average open stomata density under the stress condition was 19.6 $\pm$4. Out of 17 pea plants in control maximum open stomata density was

observed 33±2 (in ARG3 and ARG5) whereas the minimum open stomata density was observed 23±3 (in ARG10). While under treated conditions the maximum open stomata density was observed at 29 ±2 (in ARG3) whereas the minimum open stomata density was observed at 15±2 (in ARG6 and ARG10) (Table 2).

Table 2: Study the stomatal density of all treated accessions with respect to their control.

S.No.	Plant name	Open stomata in control	Open stomata in treated
1	ARG3	33 ±1.5	29 ±2.2
2	ARG5	33±1.7	28±1.3
3	ARG6	25±2.3	15±0.8
4	ARG9	28±2.5	22±2.3
5	ARG10	23±0.9	15±2.1
6	ARG13	26±2.4	16±1.3
7	ARG20	24±0.8	17±1.1
8	ARG24	30±2.3	28±1.3
9	ARG27	27±2.1	16±2.2
10	ARG31	28±1.2	21±0.9
11	ARG33	26±2.7	18±1.2
12	ARG34	29±1.2	19±1.8
13	ARG35	25±2.4	15±1.3
14	ARG37	28±3.1	16±1.2
15	ARG39	27±1.3	18±2.6
16	PS-10	26±0.9	18±3.2
17	P-3	28±2.8	20±2.2
	Average	27.4±3.3	19.6±3.8

4. Discussion

Mannitol creates the same osmotic conditions as the natural environment under stress conditions (Guo et al., 2011). In the leaf disc assay, a leaf is cut into a disc shape that represents the physiological response of plants to internal and external stimuli. It has been published previously by Chiu et al. (2021) that leaf disc assay can be used to determine stress tolerance and sensitivity in plants. In another report published by Sanan et al. (2005), a transgenic pea crop that was abiotic stress tolerant was identified using a leaf disc assay technique. Similarly in the present study, leaf disc assays were performed on different accessions of pea to identify stress-tolerant pea plants. In both studies, the leaf disc that retained its colour was said to be stress-tolerant.

Different studies have shown that number of closed stomata dramatically increased under drought stress conditions to decrease water evaporation (Caine et al., 2019). Stomata of the plants are microscopic structures which consist of a pair of specialized guard cells. An increase in turgor pressure on guard cells causes the opening of stomatal pores, which increases the rate of CO_2 uptake and water loss (Bertolino et al., 2019). Therefore, under stress conditions, the stomata attempt to close their stomatal pores for survival. In the previous study (Pirasteh-Anosheh et al. 2016), it was reported that stomata were completely closed under severe drought stress condition, these results were similar to our study in which the number of open stomata in drought-treated plants was lower in compared to control plants.

According to Arafa et al. (2021), the concentration of chlorophyll a, b and the maximum quantum efficiency of PS II (Fv/Fm) considerably decreased in pea plants under drought conditions compared with control plants. Similar results were observed in this study, the concentrations of chlorophyll a, chlorophyll b, and total chlorophyll decreased in all accessions of pea in compared to control plants however the concentration of chlorophyll a,b and total

chlorophyll were not affected severally in tolerant plants such as ARG 6, ARG10 and PS 10. The similar pattern of results was also observed in previous study in chickpea where the concentration of chlorophyll 'a', 'b' and total chlorophyll decreased 39%, 40% and 20% respectively under stress conditions (Mafakheri et al., 2010).

5. Conclusion

Leaf disc assay is a method in which the leaf is cut in a disc shape which represents the physiological response of plants to internal and external stimuli. In this study, we identify out of 17 pea plants the drought stress tolerant accession through leaf disc assay and chlorophyll content. The leaf disc result was further confirmed by stomata density. The result shows that ARG6, ARG10 (both are pea accessions) and one commercial variety PS10 exhibited better tolerance under drought conditions whereas ARG3 exhibited drought sensitivity as compared to others. In conclusion, leaf disc assay can be use as a primary method for initial identification and screening of plants for assessing their tolerance and sensitive behavior under drought stress.

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