

In Vitro Screening for Salinity Tolerance in Berseem (*Trifolium alexandrinum* L.)

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Abstract

Soil salinity has emerged as a major abiotic stressor in recent years, driven by climate change and rising temperatures. This poses a significant threat to global food security and requires adaptation and mitigation efforts. Crop improvement through breeding may help overcome salinity stress. Accordingly, we designed a study to screen berseem (*Trifolium alexandrinum* L.) varieties for salinity tolerance under in vitro conditions. We analysed the effects of high salinity (0 and 200 mM NaCl) on seed germination, shoot and root lengths, and seedling fresh and dry weight in 10 berseem varieties. Salinity significantly inhibited early seedling development, particularly seedling growth, shoots, and roots, although responses differed significantly across varieties for different traits. Data showed that Wardan showed the highest germination under saline conditions, although BL-22 and BB-2 showed comparatively less decline in dry mass. The findings indicated that salinity treatment adversely impacted plant growth more significantly than seed germination, perhaps attributable to the in vitro conditions of the study. The studied varieties of berseem were sensitive to salt stress; nevertheless, some tolerant varieties were identified.

Keywords: abiotic stress; salinity; in vitro; seed germination; plant biomass

1. INTRODUCTION

Abiotic stresses, including temperature extremes, aridity, and salinity, arise from global climate change and directly affect plant development, productivity, and quality. Soil and water salinity represent two primary constraints on agricultural yield [1]. Salinity is a major abiotic factor in dry and semi-arid regions, hindering the growth, development, and yield of commercially valuable glycophytic and halophytic crops [2]. Salinity influences various physiological, biochemical, and metabolic processes. This salinity-induced phenomenon is recognised to adversely affect agricultural output and food security [3]. The FAO (2021) reports that more than 833 million hectares of global land, constituting 10% of arable land, are impacted by salinisation, posing a significant threat to food security [4]. In India, almost 5.95 million hectares are affected by salt, with Haryana, Punjab, Rajasthan, Gujarat, and Andhra Pradesh comprising nearly 48% of the impacted soils [5].

Berseem (*Trifolium alexandrinum* L.), also known as Egyptian clover, is the predominant winter feed crop in Egypt and is essential for a sustainable agricultural system. Legumes, especially berseem, exhibit significant sensitivity to salinity, possessing a limited tolerance threshold of approximately 1.5 dS/m [6]. Berseem is an essential forage crop grown on about 2 million hectares in India, producing around 85 tons per hectare and containing 20% protein with a digestibility rate of up to 65%, making it an ideal fodder option. Elevated saline concentrations impede its development, especially influencing root length during crucial growth phases of 2-leaf, 6-leaf, and blooming, resulting in diminished yields [7]. Salt toxicity induces the buildup of reactive oxygen species (ROS) [8], such as singlet oxygen, hydrogen peroxide (H₂O₂), superoxide (O₂⁻), and hydroxyl radical (OH[•]), which can harm DNA, alter protein structures, and

cause lipid peroxidation in the cell membrane. In response to salt stress, plants activate multiple defence strategies, such as increasing antioxidative enzymes like catalase and peroxidase, to reduce ROS damage [9].

The intricate characteristics of salinity tolerance and the absence of a singular attribute for evaluation render the development of tolerant cultivars challenging and protracted [10]. Understanding the impact of salinity across berseem's growth phases is essential for developing strategies to bolster resilience in saline environments and, consequently, crop yield. Furthermore, traditional breeding methods, including hybridisation and selection in segregating populations, have not yielded satisfactory outcomes in a brief timeframe. In vitro methods allow rapid evaluation of multiple genotypes while reducing the effects of environmental stressors, including infection, temperature, and humidity [11-14].

Accordingly, this preliminary in vitro study was conducted on berseem to assess the impact of high salt concentration and identify the most salt-tolerant variety for improved breeding initiatives.

2. MATERIALS AND METHODS

2.1 Plant Material

Mature seeds of ten berseem varieties (*Trifolium alexandrinum* L.) (Table 1) were collected from ICAR-Indian Grassland and Fodder Research Institute, Jhansi, Uttar Pradesh (25°26'N and 78°35'E, 275 m above mean sea level).

TABLE 1: Description of berseem varieties used in the experiment

S.No.	Varieties	Year of release	Developed Institution
1.	JB-05-09	2005	JNKVV, Jabalpur
2.	HB-2	2014	CCS HAU, Hisar
3.	BL-43	2017	PAU, Ludhiana
4.	BL-44	2021	PAU, Ludhiana
5.	UPB-110	1993	GBPUAT, Pantnagar
6.	Wardan	1982	IGFRI, Jhansi
7.	BL-22	1988	PAU, Ludhiana
8.	Mescavi	1975	CCS HAU, Hisar
9.	BB-2	1997	IGFRI, Jhansi
10.	BL-1	1978	PAU, Ludhiana

2.2 In Vitro Salinity Study

Healthy seeds were surface sterilised in 1% sodium hypochlorite for 1 minute and rinsed three times in sterile distilled water. The seeds were sown on Murashige and Skoog (MS) medium [15] supplemented with NaCl (0 and 200 mM). Culture media were adjusted to pH 5.8 before autoclaving, then autoclaved at 121 °C for 20 min. The culture vessels with seeds were incubated in a growth chamber at 25 °C ± 1 °C, under a 16 h photoperiod with 200 µmol m⁻² s⁻¹ of photosynthetic photon flux density (PPFD).

2.3 Observations

The % germination rate was determined after 7 days of seed cultivation, while the seedling fresh weight, seedling height, shoot length (SL), root length (RL) (mm), and plant dry weight (mg) of 14-day-old seedlings were also measured.

2.4 Data Analysis

The experiment was conducted in three replicates each, with ten seeds per replication. All obtained data were subjected to a one-way Analysis of Variance (ANOVA) using XLSTAT statistical software. Differences among varieties grown under control and salinity conditions and for different traits were compared using the Tukey HSD post-hoc test at a 95% confidence level ($\alpha = 0.05$).

3. RESULTS AND DISCUSSION

3.1 Effect of NaCl on Seed Germination

The experimental results showed that in the control condition (0 mM NaCl), seed germination varied from 80 to 100% (Figure 1; Tables 2 and 3). Salt stress is one of the most significant variables influencing both vegetative and reproductive processes, affecting every phase of plant development, from seed germination to growth [16]. Salinity reduced the germination percentage in most of the varieties; the reduction ranged from 0.0 to 22.9%. Wardan maintained 100% germination under both control and salt-stress conditions. JB-05-09 (5.2%), Mescavi (7.1%), HB-2 (6.9%), and UPB-110 (10.1%) showed the least reduction, while BL-22 showed the greatest reduction (22.9%), followed by BB-2 (18.7%) and BL-1 (17.3%). Under salinity, BL-43 and BL-44 demonstrated a 12.5% decrease in germination. Researchers discovered that germination was not much influenced between 50 and 100 mM NaCl; however, it was drastically diminished at 200 mM NaCl. The adverse effect of salinity on germination is likely attributable to the reduction in water potential, leading to less water absorption by the seeds [17, 18]. Additionally, NaCl can inhibit embryo development, which also negatively affects seed germination. Many investigations of legumes showed that seedling emergence is more sensitive to salt stress than seed germination [19-21].

3.2 Effect of NaCl Concentration on Plant Growth

The studies on seedling morphology have shown that salinity affects both shoot length, root length, fresh weight and dry weight (Figure 1; Table 2 and 3). The fresh weight was significantly influenced by salinity across the varieties, exhibiting reduction range between 27.3 and 77.6%. The most significant decrease was noted in HB-2 (77.6%), followed by BL-44 (74.5%), BL-43 (74.4%), JB-05-09 (70.9%), and Mescavi (63.8%). The minimal decrease was observed in BB-2 at 27.3%, whilst BL-1 and BL-22 exhibited reductions of 55.9% and 58.2%, respectively. UPB-110 and Wardan demonstrated decreases of 61.9% and 61.7%, respectively. Salinization influences the subsequent development of plants, and its impact is contingent upon the concentration and the genetic makeup. The impact of stress on plant development is frequently assessed through the evaluation of shoot and root metrics. Growth metrics including shoot and root length, as well as biomass, are utilised to assess the impact of the stress. Similar results were reported in several crops subjected to salt stress in vitro, such as garden pea [22], grass pea [23], and tomato [24]. Salinity significantly inhibited early seedling development, resulting in a seedling length reduction of 63.0–89.4%, with BL-44 seeing the greatest decline at 89.4% and BL-1 the least at 63.0%. The length of shoots decreased by 68.0–92.9%, with the most significant reduction observed in JB-05-09 (92.9%) and the least in Mescavi (68.0%). Root length decreased by 43.8–94.1%, with the most significant reduction observed in Wardan (94.1%) and the least in BL-1 (43.8%). The dry weight exhibited a significant response, decreasing by 6.2–55.0%, with the most significant reduction in HB-2 (55.0%) and the least in BL-22 (6.2%). In general, salinity significantly inhibited seedling growth, especially affecting the development of seedlings, shoots, and roots, whereas varietal responses varied markedly across different traits.

From a physiological perspective, numerous processes are influenced by salt; however, the suppression of cellular proliferation due to water scarcity results in diminished plant growth, leaf surface area, stem extension, and overall biomass. These characteristics can serve as significant and enlightening indicators for the in vitro evaluation of salt-resistant flora [25, 26].

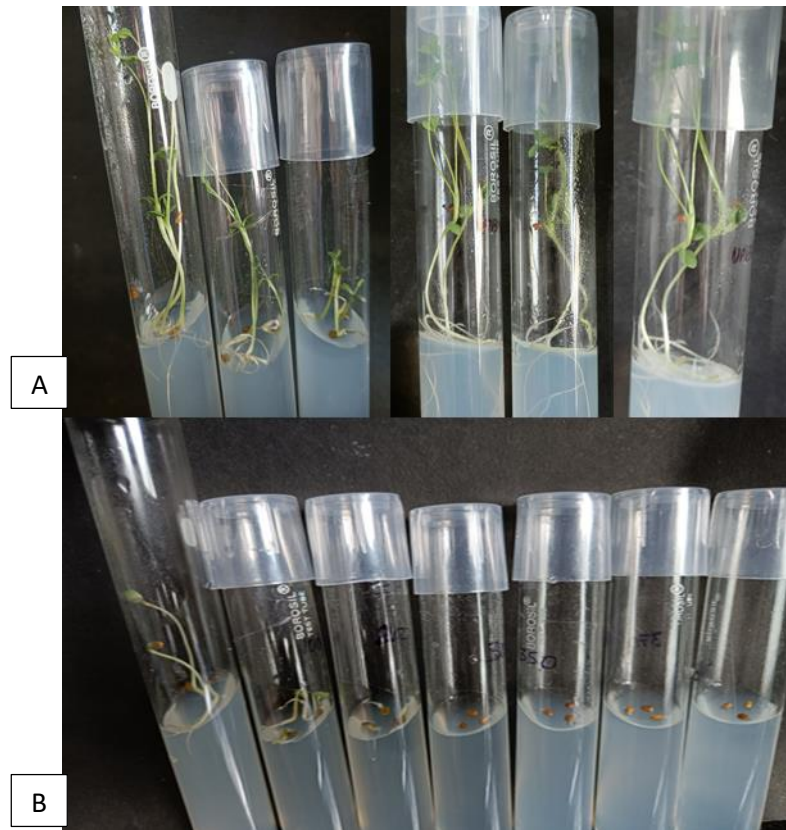


Figure 1: In vitro sowing of berseem on MS media (A) control (B) 200 mM NaCl.

TABLE 2: Percentage germination, shoot length, root length, fresh weight, and dry weight of 10 berseem varieties under control condition

Varieties	% Germination	Fresh weight (mg)	Seedling length (cm)	Shoot length (cm)	Root length (cm)	Dry weight (mg)
JB-05-09	99.95 a	146.00a	23.00 a	14.00 ab	9.00 a	9.00 ab
HB-2	80.00 c	120.50ab	20.25 ab	10.25 bc	10.00 a	10.00 a
BL-43	80.00 c	105.50bc	23.00 a	15.00 a	8.00 a	8.50 ab
BL-44	80.00 c	100.00bc	22.25 a	12.75 abc	9.50 a	7.50 ab
UPB-110	100.00 a	59.00d	21.50 ab	13.25 ab	8.25 a	7.30 ab
Wardan	100.00 a	98.00bc	19.25 ab	12.50 abc	6.75 a	7.85 ab
BL-22	98.00 a	82.50cd	18.25 ab	10.25 bc	8.00 a	6.50 ab
Mescavi	80.00 c	98.00bc	17.50 ab	8.75 c	8.75 a	8.00 ab
BB-2	90.00 b	55.00d	18.50 ab	8.75 c	9.75 a	6.00 ab
BL-1	91.00 b	80.50cd	16.50 b	10.00 bc	6.50 a	7.00 ab
C.D.	3.959	3.505	3.489	3.293	3.430	3.151
C.V.	0.098	0.290	0.138	0.209	0.286	0.202

The values indicate the mean values followed by the same letters are not significantly different at $p = 0.05$

TABLE 3: Percentage germination, shoot length, root length, fresh weight, and dry weight of 10 berseem varieties under salinity (200 mM) condition

Varieties	% Germination	Fresh weight (mg)	Seedling length (cm)	Shoot length (cm)	Root length (cm)	Dry weight (mg)
JB-05-09	94.80 b	42.50 a	3.60 a	1.00 ab	2.60 a	5.00 abc
HB-2	74.50 de	27.00 bcd	3.85 a	0.85 b	3.00 a	4.50 abc
BL-43	70.00 e	27.00 bcd	3.15 a	2.65 ab	0.60 a	5.50 abc
BL-44	70.00 e	25.50 cd	2.35 a	1.00 ab	1.35 a	5.25 abc
UPB-110	89.90 c	22.50 d	4.60 a	2.45 ab	2.15 a	3.80 c
Wardan	100.00 a	37.50 a	3.20 a	2.80 ab	0.40 a	4.70 abc
BL-22	75.55 d	34.50 abc	6.60 a	2.85 a	3.75 a	6.10 a
Mescavi	74.30 de	35.50 ab	3.70 a	2.80 ab	0.90 a	5.20 abc
BB-2	73.15 de	40.00 a	3.65 a	1.05 ab	2.80 a	5.85 ab
BL-1	75.30 d	35.50 ab	6.10 a	2.45 ab	3.65 a	4.20 bc
C.D.	3.522	3.516	3.430	3.465	3.376	3.489
C.V.	0.131	0.213	0.483	0.508	0.765	0.176

The values indicate the mean values followed by the same letters are not significantly different at $p = 0.05$

4. Conclusion

The current research revealed significant differences across varieties in their reaction to salinity stress during germination and the initial seedling phases. Salinity decreased germination, fresh weight, seedling height, shoot height, root length, and dry weight, with the most pronounced impacts typically on seedling and root development. Among the assessed varieties, Wardan showed the highest germination under saline conditions, although BL-22 and BB-2 showed comparatively smaller declines in dry mass. The varying reactions across varieties suggest a range of salinity tolerance, which can be used to select and assess salt-tolerant berseem varieties in breeding initiatives.

Conflict of interest

The authors have no competing interests to declare.

REFERENCES

- [1] Dos Santos, T. B., Ribas, A. F., de Souza, S. G. H., Budzinski, I. G. F., & Domingues, D. S. (2022). Physiological responses to drought, salinity, and heat stress in plants: a review. *Stresses*, 2(1), 113-135.
- [2] Singh, D., Kaur, A., Kaur, M., & Kumar, A. (2022). In vitro screening of Indian potato cultivars for the salt stress and associated physio-biochemical changes. *Biologia*, 77(3), 627-639.
- [3] Sanwal, S. K., Kumar, P., Kesh, H., Gupta, V. K., Kumar, A., Kumar, A., ... & Kumar, P. (2022). Salinity stress tolerance in potato cultivars: Evidence from physiological and biochemical traits. *Plants*, 11(14), 1842.
- [4] Fao, F. A. O. S. T. A. T. (2018). Food and agriculture organization of the United Nations. Rome, URL: <http://faostat.fao.org>, 403.
- [5] Mandal, A. K. (2010). Computerized database on salt affected soils in India. Bulletin (Central Soil Salinity Research Institute (Karnāl, India)).
- [6] Abd El-Naby, Z. M., Hafez, W. A. E. K., & Hashem, H. A. (2019). Remediation of salt-affected soil by natural and chemical amendments to improve berseem clover yield and nutritive quality. *African Journal of Range & Forage Science*, 36(1), 49-60.
- [7] Kazemeini, S. A., Pirasteh-Anosheh, H. A. D. I., Basirat, A., & Akram, N. A. (2018). Salinity tolerance threshold of berseem clover (*Trifolium alexandrinum*) at different growth stages. *Pakistan Journal of Botany*, 50(5), 1675-1680.

- [8] Pakar, N., Pirasteh-Anosheh, H., Emam, Y., & Pessarakli, M. (2016). Barley growth, yield, antioxidant enzymes, and ion accumulation affected by PGRs under salinity stress conditions. *Journal of Plant Nutrition*, 39(10), 1372-1379.
- [9] Ashraf, M., & Harris, P. (Eds.). (2005). *Abiotic stresses: plant resistance through breeding and molecular approaches*. CRC press.
- [10] Wang, X., Chen, Z., & Sui, N. (2024). Sensitivity and responses of chloroplasts to salt stress in plants. *Frontiers in Plant Science*, 15, 1374086.
- [11] Maamouri, A., Trifa, Y., Kouki, K., Aounallah, K., & Karmous, C. (2012). In vitro culture used for screening salt stress tolerant lettuce cultivars. *Acta Horticulturae*, 936, 477.
- [12] Raoufi, A., Salehi, H., Rahemi, M., Shekafandeh, A., & Khalili, S. (2021). In vitro screening: The best method for salt tolerance selection among pistachio rootstocks. *Journal of the Saudi Society of Agricultural Sciences*, 20(3), 146-154.
- [13] Azizi, S., Seyed Hajizadeh, H., Aghaee, A., & Kaya, O. (2023). In vitro assessment of physiological traits and ROS detoxification pathways involved in tolerance of Damask rose genotypes under salt stress. *Scientific Reports*, 13(1), 17795.
- [14] Mazri, M. A., Koufan, M., Rham, I., Radi, H., & Belkoura, I. (2023). Use of tissue culture methods to improve stress tolerance in plants. In *New Frontiers in Plant-Environment Interactions: Innovative Technologies and Developments* (pp. 425-460). Cham: Springer Nature Switzerland.
- [15] Murashige T, Skoog F (1962) A revised medium for the rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- [16] Ismail, L. M., Soliman, M. I., Abd El-Aziz, M. H., & Abdel-Aziz, H. M. (2022). Impact of silica ions and nano silica on growth and productivity of pea plants under salinity stress. *Plants*, 11(4), 494. <https://doi.org/10.3390/plants11040494>
- [17] Khosravi, Z., Pourmohammad, A., ALILOO, A. A., Shahabivand, S., Hassanpouraghdam, M. B., & Topcu, H. (2022). In vitro salinity stress mediates grass pea genotypes'(Lathyrus sativus L.) responses. *Turkish Journal of Agriculture and Forestry*, 46(3), 340-351. <https://doi.org/10.55730/1300-011X.3007>
- [18] Pandit, K., Kaur, S., Kumar, M., Bhardwaj, R., & Kaur, S. (2024). Salinity stress: Impact on plant growth. In *Advances in food security and sustainability* (Vol. 9, pp. 145-160). Elsevier. 10.1016/bs.af2s.2024.07.002
- [19] Tokarz, B., Wójtowicz, T., Makowski, W., Jędrzejczyk, R. J., & Tokarz, K. M. (2020). What is the difference between the response of grass pea (Lathyrus sativus L.) to salinity and drought stress?—A physiological study. *Agronomy*, 10(6), 833.
- [20] Grozeva, S., Kalapchieva, S., & Tringovska, I. (2023). In vitro screening for salinity tolerance in garden pea (Pisum sativum L.). *Horticulturae*, 9(3), 338.
- [21] Johal, N., Sonkar, I., Kaur, H., & Kaur, S. (2025). Exploring salt sensitivity in berseem (Trifolium alexandrinum) through analysis of ROS-scavenging attributes. *The Indian Journal of Agricultural Sciences*, 95(12), 1499-1503.
- [22] Ehtaiwwesh, A. F., & Emsahel, M. J. (2020). Impact of salinity stress on germination and growth of pea (Pisum sativum L.) plants. *Al-Mukhtar J. Sci*, 35, 146-159.
- [23] Ishrat, F., Afrasiab, H., & Chaudhury, F. A. (2022). Effects of salinity stress on growth and physio-biochemical parameters of three pea (Pisum sativum L.) cultivars of different maturity duration. *Advancements in Life Sciences*, 9(3), 380-391.
- [24] Piwowarczyk, B., Tokarz, K., & Kamińska, I. (2016). Responses of grass pea seedlings to salinity stress in in vitro culture conditions. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 124(2), 227-240.
- [25] Zaki, H. E., & Yokoi, S. (2016). A comparative in vitro study of salt tolerance in cultivated tomato and related wild species. *Plant Biotechnology*, 33(5), 361-372.
- [26] Attia, H. (2023). Physiological Responses of Pea Plants to Salinity and Gibberellic Acid. *Phyton* (0031-9457), 92(1).

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