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Cytological impact and seed germination responses of salts (NaNO3 and KNO3) stress on Barseem (*Trifolium alexandrinum* L.)

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Abstract
Berseem (*Trifolium alexandrinum* L.) belongs to the fabaceae family and it is one of the most important *ravi* fodder legume crops in northern India. The high digestibility, along with palatability, superior forage quality, long duration of green fodder availability have made it best kind of fodder. Because of its water requirement, this crop can only be grown in irrigated areas; hence seed germination is impacted by water availability and environmental stress. In the present study dry and healthy seeds of Berseem (variety Wardan) were germinated under varying NaNO3 and KNO3 salt, concentration range of 0 mM, 50 mM, 100 mM, and 150 mM. It was observed that appearance of radical, plumule and cotyledonary leaves significantly affected by salt NaNO3 and KNO3 at 100mM concentration. Our experiment reveled that salinity affected cytological process; the highest MI values were obtained from control *viz*., 10.32±0.26^{a} whereas MI values were declined with increasing the concentration of salts. A broad spectrum of chromosomal abnormality *viz*., stickiness, scattering, precocious movement of chromosome, unoriantation, loop formation, laggard and forward movement were reported. Tab% was maximum (4.80±0.02) at the concentration of 150 mM KNO3 followed by maximum (4.70±0.30) in case of NaNO3 at same concentration, it was gradually increased with increasing the concentration.

Keywords: KNO3, MI %, NaNO3, Tab%, *Trifolium alexandrinum*

Introduction
Abiotic stress is an unfavorable condition that influencing plants metabolism, homeostasis, growth, development, crop yield etc. World- wide and affects 20% of irrigated land [1]. Salinity is one of the abiotic stress which initiates water shortfall even in appropriately watered soils by diminishing the osmotic ability of soil solutes making water extraction from the soil tough for roots by causing restraint impacts [2, 3]. Sinha *et al*., 2018 [4] previously reported that its impact on morphological, physiological and internal transformational modifications depends upon the concentration of the salt, treatment time. Primarily, salinity affects roots of plants which causes inhibition of water uptake, cell expansion, lateral bud development and death of the plant [5] along with this the saltiness likewise articulates the ionic pressure causing ions aggregation and accumulation in cells which causing chlorosis, nacrosis and decrease in sub-cellular photosynthetic exercises prompting early senescence[6]. Munns and Tester 2008 [5] reveled that, roots act as the primary receptor of the salt stress that's why roots are most reliable system to examine the cellular and molecular mechanism. High concentration of salts causes plasma membrane deformation, DNA fragment discontinuity and incense deformation in cells and its organelle. Earlier Lin *et al*. 2006 [7] and Li *et al*. 2007 [8] reported that salt stress induced DNA fragmentation in barley roots where as in root cells of rice has been programmed cell death. The accumulation of ions can limit the germination and development of several species that lead to morphological, biochemical, cellular and molecular alterations [9, 10]. Saltiness doesn't make consistently antagonistic impacts rather low salt concentration may increases flowering and induced longer root in plants [11]. DeWald *et al*. 2001 [12] reported some anti-apoptotic genes such as Bcl2 or SOS1 in Arabidopsis, overexertion of this gene prevent salt-mediated DNA fragmentation and apoptosis. Present piece of work has been performed with an intend with the impact of salts (NaNo3 and KNo3) on germination and cytological variation in *Trifolium alexandrinum* L. It is well known green feed and discovers its application because of characteristics like simple edibility, satisfactoriness and richness of mineral substance [13].

Material and methods
Seeds procurement- and viability percentage determination
T. alexandrinum variety Wardan was procured from Indian Grassland and Fodder Research Institute, Jhansi, Uttar Pradesh, India. Subsequent to getting the seeds, were starilized with 10% sodium hypochloride for 10 minutes and were then soaked in distill water. Viability of individual was accessed by germinating 30 seeds in blotting paper moist with sterile distilled water and kept on Petridis.

Germination of seeds under varying salinity stress
Thirty seeds of *T. alexandrinum* variety Wardan were germinated under NaNO3 and KNO3 varying concentrations. The procedure described by Sinha *et al*., 2018 was followed during seeds germination under controlled condition along with this the concentration of salt chosen for the study were 0 mM, 50 mM, 100 mM, and 150 mM which was based upon previous research articles [14, 15]. Observed the appearance of radical, plumule and cotyledonary leaves at regular intervals of 24 hours a day. The experimental sets were having three replicates for statistical validation.Seed germination percentage (G %) was expressed by following formula-

G % = (Number of germinated seeds/Number of experimental seeds) * 100.

Cytological study

For assessment of cell divisions, the roots were pre-treated with 2 mM, 8-hydroxyqunoline for 3 h, fixed in 3:1 ethanol: acetic acid (v/v),then hydrolyzed for 2 min in 1 N HCl at 60 °C and stained in 2 % aceto-carmine. Root tips of 1 mm beginning from the tip were analyzed and crushed [16]. For every treatment 3 squash arrangements were made; near about 850 cells were scored for each slide under a binocular light magnifying instrument (Olympus BX 51). MI% and TAb % were scored by the following formula;

MI%= No. of dividing cell/ total no. of observed cell*100

TAb%= Total No. of abnormal cell/ Total No. of observed cell *100

Statistical evaluations- The data was analyzed by the SPSS 16.0 software (1968). There were three replicates of plants for each treatment and one independent variation. A one way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT, $P < 0.05$) were performed for mean separation and the graph was plotted using Sigma Plot 10.0 software (Systat Software Inc.). Actual means and standard errors were calculated.

Result

The germination rate more than 93% was accomplished 10 day time from soaking the seeds of *Trifolium alexandrinum* ver. Wardan in water. This showed that the seeds are of good viability for germination. The radical protrusion (G_r %) of the control sets were reported maximum 93.6±0.37 %. As the concentration of NaNo3 salt were increased, radical emergence declined up to 73.30±0.06 % (150mM). Salt KNo3 showed that 100 mM is the stimulatory dose for radical protrusion having 91.4±0.46 % emergence which drastically decreased to minimum 71.1±0.76 % at 150 mM concentration (Fig. 1). The rate of emergence of plumule (G_p %) was found dependent on the number of the radical protrusion. In the controlled set it was reported 82.2±0.44 % which were stimulated by the treatment of the 50mM NaNo3 salt concentration, whereas G_p % showed declination by increasing KNo3 salt concentration (Fig. 1). Among these salts and the doses 150 mM of KNo3 showed minimum 48.4±0.61 G_p %. The rate of emergence of cotyledonary leaves (G_c %) were depends on the emergence of plumule. G_c% were found to maximum 86.0±0.32 in controlled sets. These results reviled that as concentration of salts increased G_c% decreased. 100 mM NaNo3 showed more inhibitory concentration after that declination rate was found less than 50% (40.9±0.39) but in the treatment of KNo3, declination rate (34.8±0.30) was found at 150mM concentration (Fig. 1).

Effect of salt stress on active mitotic index (MI) -The effect of salt stress on MI and TAb (%) was summarized in Table 2. According to the results, MI significantly decreased in different concentrations of salts in comparison to respective controls. The highest MI values were obtained from control *viz*., 10.32±0.26[a] whereas applications of 50 mM *NaNo3* with a score of 8.68±0.21[c], as comparison to this salt, *KNo3* with a score of, 9.44±0.13[b.] Minimum (5.47±0.09) MI % were reported in 150mM KNo3 where as at same concentration of the salt NaNo3 has been slightly more MI % as 5.70±0.29.

Effect of salt stress on chromosomal abnormalities- Table 1 shows various types of chromosomal abnormalities along with a decrease in the mitotic index. The observation showed that mitotic abnormalities increased with increasing concentration. All NaNo3 and KNo3 concentrations could induce different types of chromosomal abnormalities. Anomalies like stickiness (Fig. 2 C), Scattering (Fig. 2 C), Precocious movement (Fig. 2 G), unoriantation (Fig. 2 H), loop formation (Fig. 2 D), forward movement (Fig. 2 I) and other abnormalities were observed. Stickiness in treated cells was major concern in the salt treatment and it was found in all treatment sets (Table 1). Scattering was found to higher doses of salts. Precocious movement was found minimum 0.18±0.09 at 50 mM NaNo3 concentration which increased up to 0.35±0.09 at highest dose where as in KNo3 salt treatment it was reported that lower dose (50mM) have been maximum 0.37±0.10. Loop formation is the key reporting in this treatment, it was found that loop were present in all the concentration. NaNo3 treated set showed Forward movement of chromosome at all concentration where as in KNo3 treated set at lower dose not to be observed (Table 1). In this results, TAb % were found significantly increase by increasing the concentrations of both salts. At the concentration 50mM of KNo3, TAb % was minimum 1.58±0.13 which drastically increase to 3.73±0.30 and 4.80±0.02 at 100mM and 150 mM concentration respectively. In comparisons to KNO3, NaNo3 showed similar trend which increased to 1.84±0.88 to 4.7±0.13 at the concentrations 50mM to 150 mM respectively.

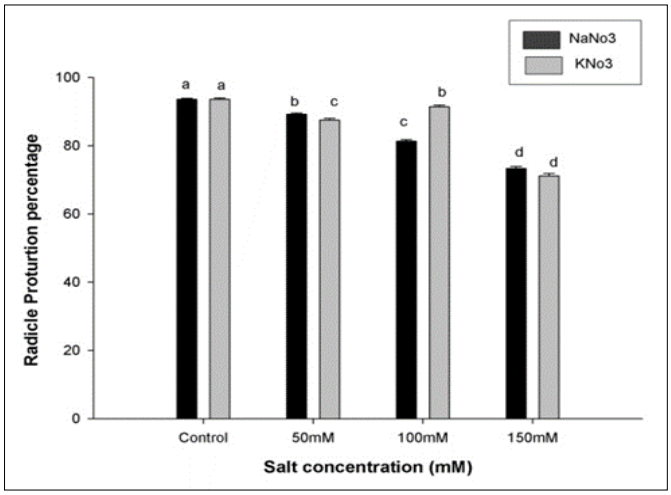

Fig 1(a): Comparative account of radical proturtion (Gr%) for NaNo3 and KNo3 salts

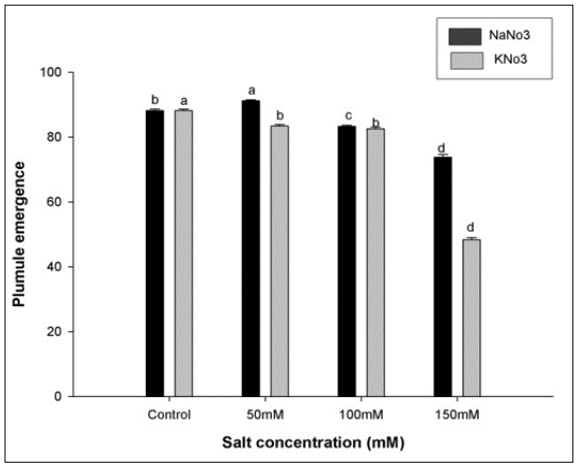

Fig 1(b): Comparative account of Plumule emergence (Gp %) for NaNo3 and KNo3 salts

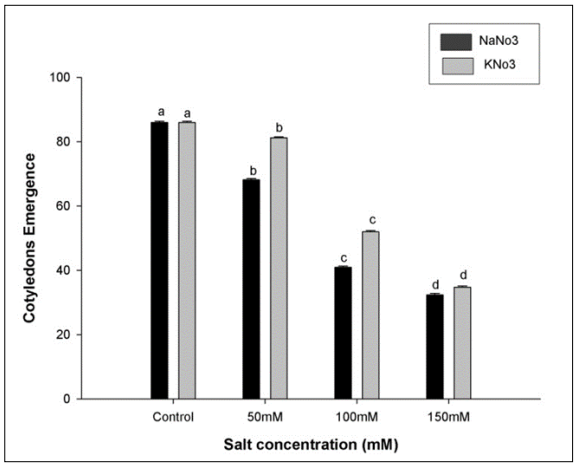

Fig 1(c): Comparative account of cotyledons emergence (Gc %) for NaNo3 and KNo3 salts

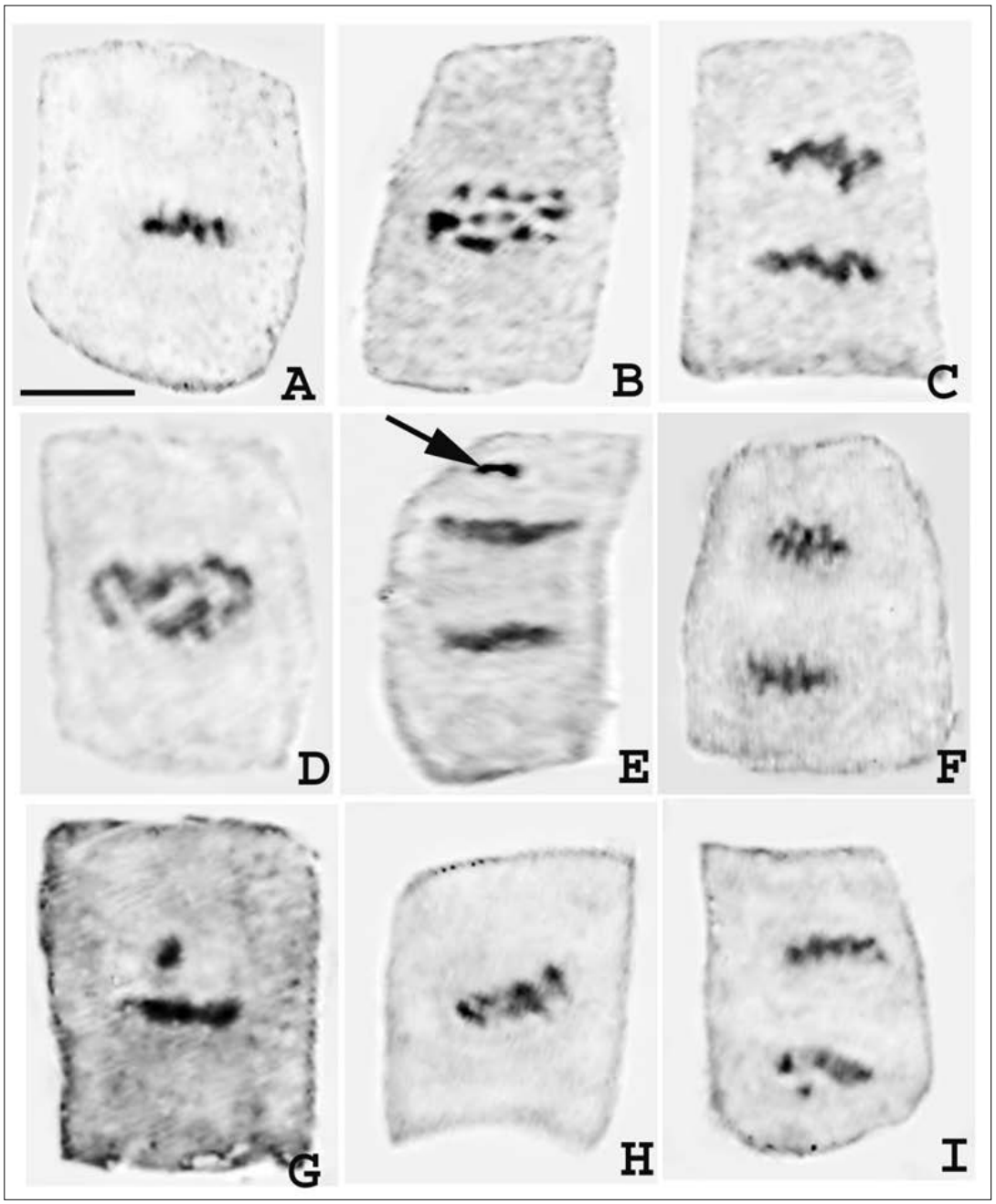

Fig 4: Metaphase, B. scattered Metaphase, C. Stickiness at anaphase, D. Loop formation, E. Forward movement, F. Normal anaphase, G. Precocious movement, H. Unorientation metaphase, I. Forward movement with unorientation of anaphases.

Table 1: Showing the account of AMI (%), TAb (%) and various abnormalities induced by salt NaNo3 and KNO3 in root meristems of *Trifolium alexandrinum* L.

Salt	Salt Conc.	AMI	Metaphasic Abnormalities					Anaphasic abnormalities					Oth	TAb
			St	Sc	Pr	Un	Lf	Asc	Ast	Aun	Lg	Fw		
NaNo3	Control	10.32±0.26[a]	-	-	-	-	-	-	-	-	-	-	-	-
	50mM	8.68±0.21[c]	0.28±0.16		0.18±0.09	0.18±0.09	0.28±0.16	0.27±0.16	-	0.27±0.15	0.18±0.09	0.18±0.09	-	1.84±0.88
	100mM	6.98±0.36[d]	0.35±0.08	0.17±0.08	0.27±0.16	0.62±0.08	0.27±0.16	0.17±0.08	0.27±0.16	0.37±0.10	-	0.25±0.14	0.35±0.07	3.14±0.24
	150mM	5.70±0.29[e]	0.53±0.14	0.36±0.23	0.35±0.09	0.79±0.41	0.26±0.15	0.36±0.24	0.45±0.24	0.36±0.23	0.44±0.08	0.42±0.30	0.36±0.09	4.7±0.13
KNO3	Control	10.32±0.26[a]	-	-	-	-	-	-	-	-	-	-	-	-
	50mM	9.44±0.13[b]	0.27±0.16	-	0.37±0.10	-	0.37±0.10	-	0.27±0.15	-	-	-	0.28±0.17	1.58±0.13
	100mM	6.65±0.05[d]	0.51±0.26	0.27±.015	0.18±0.09	0.94±0.13	0.28±0.17	0.18±0.09	0.28±0.17	0.39±0.26	0.18±0.09	0.18±0.09	0.29±0.16	3.73±0.30
	150mM	5.47±0.09[e]	0.49±0.25	0.28±0.16	0.30±0.18	0.69±0.13	0.49±0.10	0.37±0.18	0.49±0.25	0.40±0.20	0.37±0.24	0.40±0.20	0.46±0.24	4.80±0.02

Discussion

Seed germination is the most sensitive phase for vegetative plant growth, the salt content is a crucial condition for the viability of the seeds and reduces seed vitality, delays seed germination and influences the quality of the forage [17, 18, 19, 20] It was found that the salt concentration did not significantly affect the germination of the seeds up to a range of 50mM, but by increasing the concentrations to 100mM and 150mM, the salt content began to inhibit germination, which is similar to the results obtained in plants such as Nicotiana, wheat, cucumis etc [21, 22]. Salinity can have initial phases of action and can show changes in the water absorption capacity of root systems, changes in the water loss of the leaves and effects as hyperosmotic stress [5]. This study was conducted to evaluate possible changes in germination and cytological activities under saline stress conditions. The mitotic index are a reliable parameter for estimating the frequency of cell division [23]. Changes in mitotic activities and their inhibition are generally used to measure cytotoxicity. In the present study, mitotic activity was reduced in both salinized plants, suggesting that salt stress resulted in a decrease in the number of cells entering into mitotic division. The concentration- and time-dependent inhibition of MI describes the cytotoxic potential of the salt in A. cepa.

The significant reduction in MI may be the result of the mito depressant effects of salt, which disrupt the normal mitotic process, resulting in a decrease in the number of cells dividing. The response to salt stress involves inhibiting cell production by inhibiting the cell cycle and reducing meristems [24]. In addition, it may be due to the inhibition of protein synthesis, DNA synthesis or the effect on the S or G2 / M phase of the cell cycle and the reduction in cyclin-dependent kinase (CDK) activity [24]. The finding of a decrease in the MI and an increase in the TAb% value was directly proportional to the severity of the mitotic inhibition [25]. In addition, the use of these salts also changed the frequency of mitotic phases. In this study, different types of chromosomal abnormalities were identified, among which five main types of chromosomal aberrations were observed in different mitotic phases: stickiness at metaphase and anaphase, precocious movement, unorientation at meta and anaphase, loop formation at metaphase and forward movement. Chromosome stickiness is viewed as a chromatid-like aberration [26] that causes shortened and thickened chromosomes in prophase and metaphase. Precocious chromosome movement seen during metaphase might be caused by early chiasma terminalisation or univalent chromosome creation at the conclusion of prophase. Unorientation may occur as a result of spindle structure disruption, resulting in spindle fibre imbalance on both sides of the centromere traction power, however chromosomal acentric fracture cannot produce normal chromosome movement [27]. The formation of loops during metaphase may have resulted from the inability of kinetochores to adhere to spindles, resulting in the merging of ends and the formation of loops. Such conditions can result in mutations. This means that in terms of chromatin organization, KNO3 and NaNo3 can be linked to the influence on the physical and chemical properties of DNA and proteins or both and leading to improper folding of chromatin [28].

Conclusion

In summary, salinization has a chromotoxic effect on seedling cells, which inhibits DNA, core protein synthesis, compatible osmolytes, antioxidant defense enzymes, polyamines and limits the availability of water [29]. Apart from that, it causes sensitive oxygen species, ion toxicity, improves alkalinity and osmotic pressure, disrupts metabolic mechanism, cell physiology and ion transport, which together increase the types of chromatin abnormalities in cells [30]. The effect of stress on seedlings depends on the chemical and physiological nature of stress-inducing compounds such as KNo3 and NaNo3. Both the salt has an ionic nature and induces ionic toxicity in the cytosol of the cell. Cytological abnormalities in the roots of the seedlings, which in higher doses negatively affect the growth and development of the plant. Therefore, further studies related to gene expression are needed to improve Berseem under saline conditions.

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Conflict of interest statement

The authors declare no conflict of interest.

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