Salt Tolerant *Gossypium hirsutum* L. Cultivars are Mostly Long-Staple Length Cultivars

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Research Article

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Abstract

Background: Cotton is a major cash crop in the global and, in particular, the Indian markets, playing an important economic role in the textile and oil industries. The cotton plant is one of the highly bred plants that is highly sensitive to salt stress. As cotton is a non-food crop, the availability of non-saline terrain and water for the cultivation of cotton plants is only next to other food crops, thereby posing a need to better understand the salt tolerance of this plant. *Gossypium hirsutum* L. cultivars MCU 5, LRA 5166, and SVPR 2 were selected based on exomorphic traits like staple length and cropping season so that the genotypic responses to salt stress and salt shock can be compared for interpreting the effects of salinity on *in vitro* germination. Thus, this study aims to establish genotypic dependence on salinity tolerance.

Results: The results affirmed genotypic variation in salinity tolerance, with MCU 5 tolerating salt stress better than LRA 5166 and SVPR 2 in all the observed stages of growth of the plant and the parameters measured. Further salt-tolerant cotton varieties were observed to be long-staple length varieties; staple length is the fiber character of the cotton lint. Moreover, salt tolerance in the vegetative growth stage of cotton plants is not independent of the germination stage of the plant.

Conclusion: Nevertheless, the correlation of genotypic dependence to morphological characteristics, in particular, staple length (and cropping season), is of agronomic and commercial significance. Further research by screening and investigating a greater number of cultivars using biochemical and molecular techniques will provide a better understanding of this observed phenotypical relationship to the genotypes of cotton cultivars under salt stress.

Background:

Gossypium hirsutum L. is a salt-sensitive glycophyte of high commercial importance, influencing both the agriculture and the textile industries. In India, all the four useful species of the cotton plant, *Gossypium hirsutum* L., *Gossypium arboretum* L., *Gossypium herbaceum* L., and *Gossypium barbadense* L., are cultivated. Bt cotton, a genetically engineered variety, is also cultivated. Cotton cultivation in India is spread mainly on 65% of the dry land and only on 35% of the irrigated land. These agricultural lands were recently estimated to be under threat and vulnerable to salinity as published in a report by UNU-IWEH (Qadir et al. 2014).

Due to inconsistency (Wang et al. 2007), evaluation of *in vitro* germination for salt tolerance in cultivars of *Gossypium hirsutum* L. has been debated for a long time as an authenticated rapid screening technique (Almansouri et al. 2001). However, the difficulty of screening large populations using other methods makes this the preferred method. Needless to mention, *in vitro* germination turned out to be the technique used for the evaluation of the effect of salinity toxicity on cotton cultivars in this study.

While most of the studies discuss the impact of salinity on root development (Fuxin et al. 2000; Kent and Lauchli, 1985; Cramer et al. 1985; Cramer et al. 1987; and Jun-Juan et al. 2007), only a little information is available on the implications of salinity on shoot development and germination (both of which are best understood under *in vitro* conditions). Furthermore, Ungar (1995) also stated that *in vitro* germination of cotton seeds needs to be assessed in two ways: germination under varying saline concentrations and germination of salt-shock pre-treated cotton seeds. Inhibition of imbibition and seed germination was attributed to osmotic and water stress (components of salt stress), which in turn impacts the mobilisation of reserved forms of energy required for germination (Lin and Kao, 1995) and inhibits processes related to protein synthesis (Ramagopal, 1990).

Hence, this study investigates the plausibility of confirming genotypic variation in salt tolerance amongst *Gossypium hirsutum* L. cultivars. Cotyledonary embryos of the selected cultivars were tested for salt tolerance using *in vitro* techniques on germination medium supplemented with salt at different concentrations and by pre-treating cotton seeds with the same salt concentrations as in the salt supplementation experiment before cultivation on germination medium without salt supplementation. Time and growth parameters of germination and shoot development were measured to assess salinity tolerance.

Results:

Under salt stress, the germination efficacy of the chosen cultivars of *Gossypium hirsutum* L., MCU 5, SVPR 2, and LRA 5166 was measured as parameters of time and growth. Imbibition and radicle emergence against time, growth parameters like hypocotyl length and mean shoot length, and visual interpretation of greening of the cotyledon was the selected criteria for studying the impact of salinity on *in vitro* germination of cotton.

The results were consistent, clearly demonstrating that the impact of salinity varies within the genotypes of cotton plants. While the measured components of time on all the three cultivars remained consistent at all salt concentrations; variations observed in growth characteristics within the three cultivars imply the effects of salt stress.

The trend in germination percentage did not vary between the two types of salt treatment in the genotypes, MCU 5 and SVPR 2, but an opposing trend was observed in the cultivar LRA 5166 (Figure 1 and 2). However, MCU 5 had increased salt tolerance, in fact, favouring increased survivability in the presence of higher salt concentrations by up to 80%, while SVPR 2 had a survival rate falling by approximately 40%. In contrast, LRA 5166 tolerated salt shock treatment of 0.01 mM NaCl with a survival rate increasing to 70%, which decreased to 50% when grown in a medium supplemented with a salt concentration of 1 mM.

Visual measurements of the greening of the cotyledons had no effect on all the genotypes and were independent of salt shock treatment, with only LRA 5166 showing a reverse effect in variations. In NaCl supplemented medium, MCU 5 and SVPR 2 showed a decrease in greening patterns with increasing salt concentrations; contrarily, LRA 5166 showed an increase in greening patterns with increasing NaCl concentrations.

Table 1 clearly shows that MCU 5 responds to salt shock treatment with a threefold increase in mean shoot length, a common physiological elongation response of shoots to increased NaCl concentrations; LRA 5166 also demonstrated some salt tolerance, but SVPR 2 succumbed to concentrations greater than 0.1 mM NaCl in salt shock pre-treatment experiments. These responses suggest that salt shock pre-treatment of cotton seeds will result in an exchange of ions during imbibition before radicle emergence, and the effect of NaCl at this stage of imbibition and radicle emergence will reflect on the later vegetative growth stage of the *Gossypium* sp. plants.

As observed in Table 2, NaCl stress by supplementation made it obvious that MCU 5 was the only cultivar that tolerated higher NaCl concentrations, while LRA 5166 and SVPR 2 succumbed to salt stress, in the order of their salt tolerance. SVPR 2 was more sensitive to salt stress than LRA 5166 and MCU 5 at the highest 1 mM NaCl concentration.

Discussion:

For the interpretation of seed germination responses to salt stress, cotton cultivars were imposed on two types of NaCl treatments, which had already been reported by Ungar (1995). He emphasised that this kind of study will reveal important findings of variations in plant growth patterns when germinating under higher saline concentrations and when germinating after recovery from high salt shock exposure. This field-level study in cotton plants for recovery following salt stress showed that the cotton plants were much more tolerant of salt stress at the boll development stage and least tolerant at seed germination and the vegetative stage of growth. A similar comparative interpretation of the results from this study, as in Tables 1 and 2, infers that cotton seeds do not have the ability to recover from salt shock in the vegetative growth stage of the plant. This is concluded from the growth parametric results of the cotton plants showing the impact of salt shock being carried forward to the vegetative stage of the plant as a symptomatic increase in mean shoot length (mid-stem elongation), unlike the absence of such variations in mean shoot length when salt was supplemented in a concurrent experiment using the

same cultivars and imposing the same salt concentrations.

Table – 1: *In vitro* Germination after Salt Shock Treatment for 48 Hours

Salt Concentration in mM	Cultivar	Hypocotyl Length (mm)	Mean Shoot Length (mm)	Cotyledon Greening
Control	MCU 5	0	0	++++
0.01		20	29	++++
0.1		30	41	++++
1		45	79	++++
Control	LRA 5166	30	39	++++
0.01		15	19	++
0.1		0	0	+++
1		34	48	+++
Control	SVPR 2	28	37	++++
0.01		18	20	+++
0.1		37	56	++++
1		15	17	++++

Table – 2: *In vitro* Germination on Salt Supplemented Medium				
Salt Concentration in mM	Cultivar	Hypocotyl Length (mm)	Mean Shoot Length (mm)	Cotyledon Greening
Control	MCU 5	82	89	++++
0.01		83	86	++
0.1		83	88	+++
1		88	93	+++
Control	LRA 5166	95	98	+
0.01		65	70	+++
0.1		80	84	+++
1		60	63	++++
Control	SVPR 2	92	98	++++
0.01		78	83	+++
0.1		59	62	+++
1		0	0	NA

Moreover, prior studies on *in vitro* germination of *Gossypium hirsutum* cultivars showed that salinity toxicity predominantly affected root development. In a study by Wang et al. (2000), NaCl stress was observed to deteriorate germination rate, resulting in decreased root growth, cotyledon size, and dry weight. Similarly, in a short communication by Kent and Lauchli (1985), they attributed increased ion toxicity caused by Na^+ ion accumulation to decreased root growth, which was not altered by Ca^{2+} amelioration, unlike the alleviation in root growth that was observed by reducing the efflux of K^+ ions. This suggested that the observed variations in plant growth patterns in this study are because of Na^+ ion accumulation in shoots and root development was directly correlated to shoot development and vice versa. Furthermore, from this study, it was reaffirmed that salt stress impacted root development *in vitro*.

In light of these germination studies in cotton, two new research areas explored the interposition effects of two main germination hormones: gibberellin and abscisic acid. A recent study by Chen et al. (2021) and Zhang et al. (2021) explained that salt stress affects cotton seed germination by the upregulation of ABA and the downregulation of GA, if not for the priming of seeds using melatonin, a commonly used stress-alleviating chemical that interposes the upregulation of GA expression and the downregulation of ABA hormones. Further, a study by Zhao et al. (2020) showed that the use of auxin priming for cotton seeds enhanced seed germination. Thus, the existence of alternate biochemical pathways that interpose the hormone signaling during germination indicates the possibility of an external physical interventional strategy to enhance seed germination and also the need to understand the complex and extensive mechanisms of salt tolerance that can be exploited for breeding improved salt-tolerant varieties.

Further interpretation by comparing the variation of salt tolerance with the genotype characteristics provided deeper insights into the observed genotype dependency of cotton varieties for salt tolerance. The most salt-tolerant *Gossypium hirsutum* cultivar, MCU 5, is a summer crop with a long staple length. The crops succumbing to salt stress are LRA 5166, a winter and a drought-resistant crop with a medium staple length; and SVPR 2, a monsoonal crop (the chances of dilution of salinity are high and thus tolerance to salinity is low) with a medium staple length. A critical perspective on the genotype screening study by Chaudhary et al. (2020) for salt tolerance in cotton varieties showed that all the identified salt-tolerant varieties are of long or superior long staple length. When research results on the staple length category of the salt-tolerant cultivar identified in various publications to date were consolidated into Table-3, it was observed that all the cotton cultivars screened as salt-tolerant cultivars, including the salt-tolerant cultivar screened in the current study, were cotton cultivars with long, superior long, or very rarely medium staple length. This is the first research to systematically report the observed relationship between long staple length cotton cultivars and salt tolerance by collating the findings from previous and current research (Table 3).

Table – 3: Salt Tolerant Genotypes and Staple Length Type			
Manuscript Citation	**Year**	**Salt Tolerant Genotypes**	**Staple Length**
Chaudhary MT et al.	2020	NIAB-545	superior long
		CIM-595	superior long
		Coker-307	Long
		FH-113	long
		FH-942	long
		DNH-40	long
Moussourak MA et al.	2019	Hersi	superior long
		KEHKSHAN	?
		S-3	short
Farooq et al.	2019	NIAB-824	superior long
		MNH-988	superior long
Yadav et al.	2017	JK-4	superior long
		PH 1009	Medium
		RDT-17	?
Bibi et al.	2016	CIM-707	superior long
		CIM-446	superior long
Ibrahim et al.	2017	Zhongmian 41	long staple

Yet, this observation of long-staple length cotton cultivars always being salt-tolerant does not necessitate the conclusion that all long-staple cotton cultivars are salt-tolerant or that staple length influences salt tolerance in cotton cultivars. However, molecular and genomic studies led Feng et al. (2021) to hypothesise that the expression of the gene *GhCalSs* in cotton seed fibers and during stress responses indicates the influence of stress on fiber formation. *GhCalSs* gene codes for callose synthase enzyme which is involved in callose synthesis for fiber formation in cotton seeds. Similarly, Wang et al. (2021), while discussing their viewpoints on cotton fiber initiation being regulated by sugar signaling due to the interaction of the MBW transcription factor complex and auxin signaling, contemplated MBW complex looping during a stress response. Thus, there is very little advancement in knowledge when it comes to genome-wide studies relating to cotton fiber formation and salt stress responses. In another study, Lv et al. (2021) found 93 *expansin* genes expressed distinctly during different stages of fiber development in *Gossypium hirsutum* and Zheng et al. (2021) found 27 SNP markers relating to salt tolerance traits, indicating that fiber development in cotton can have genomic relationships to genes expressed in response to salt stress. This research, following the outcome of this research relating this phenotype, staple length or fiber length, to genotypes of cotton for salt tolerance, indicates that there is scope for advancement in this light by investigating the genomics of fiber length (and so the staple length type) of cotton genotypes contributing to the salt tolerance of the plant. This kind of study across a greater number of cotton cultivars using genomic techniques to search for patterns relating to staple length and salt tolerance can help in developing mass screening methods.

Conclusion:

Thus, this study clearly shows that there is a genotype-dependent variation in salt tolerance, with MCU 5 being the most tolerant of the three chosen cultivars, followed by LRA5166 and SVPR 2. The same pattern can be seen in germination percentage, with MCU 5 being a more salt-tolerant genotype than LRA 5166 and SVPR 2. Further, this genotype-dependent variation in salinity is phenotypically related to the staple length and probably the cropping season. To date, the identified salt-tolerant cultivars in cotton are from the long staple length genotypic category. In addition, cotton seeds do not recover from salt shock given at the time of germination. This salt shock treatment impacts the vegetative stage of the plant, affecting the mean shoot elongation as opposed to the observed growth of the plant when salt stress was imposed directly on the vegetative stage of plant growth. This in turn implies that the salt stress effect carried forward from the germination stage to the vegetative growth stage of the cotton plant is more overwhelming than the effect of salt stress imposed directly during the vegetative growth stage of the plant.

The results of this study need to be affirmed further by increasing the scope to include other available varieties and by using biochemical analyses. The research can also be extended to find clues that relate salt tolerance with staple length (and cropping season) at the molecular level using molecular analyses and bioinformatics tools.

Methods:

1.1 Procurement of Plant Material:

Authenticated delinted seed samples of selected varieties, MCU 5, LRA 5166, and SVPR 2, were purchased from registered seed shops. The genotypes used differed depending on the season of cultivation and the length of the staple. MCU 5 is a winter crop with a long staple length; SVPR 2 is a monsoonal crop with a medium staple length (often commercially short); and LRA 5166 is a summer crop with a medium staple length.

1.2 Culture Medium:

Murashige and Skoog medium (1962) composed of major and minor nutrient salts, vitamins, sucrose (30 g l^{-1}), and meso-inositol (0.1 g l^{-1}) were prepared with the addition of 0.5 ppm 2, 4-D hormone regulators and sterilised at 121° C for 10 to 15 minutes. Salt as NaCl was supplemented at concentrations of 0.01

mM, 0.1 mM, and 1 mM before sterilisation. Triplicates for each treatment were prepared along with the control (without NaCl supplementation).

1.3 Preparation and Inoculation of Culture Material:

Seeds were first washed in soap water for 5 minutes. This was followed by surface sterilisation of seeds in Bavistin fungicide (adopting instructions on the package) for 3 minutes; agitated with 0.05% mercury chloride for 3 minutes for MCU 5, 5 minutes for LRA 5166, and 3 minutes for SVPR 2; and washed five times in sterile distilled water.

Surface sterilised intact seeds were soaked in sterile distilled water and sterile saline of varying concentrations (0.01 mM, 0.1 mM, and 1 mM) for 48 hours before inoculation. The seed coat was removed after 48 hours to isolate cotyledonary embryos with emerging radicles under aseptic conditions in the inoculation chamber. These salt-shock pre-treated cotton explants, along with the ones soaked only in sterile distilled water, were inoculated in standardised MS medium without NaCl.

Concurrently, cotton cotyledonary embryos were surface sterilised with 0.5% alcohol before being inoculated in standardised MS medium supplemented with similar NaCl concentrations (0.01 mM, 0.1 mM, and 1 mM), as well as a control with no NaCl supplementation. Inoculation was carried out under sterile conditions in a sterile chamber.

All explants were rinsed in undiluted alcohol before being transferred to culture tubes. The inoculated tubes were incubated for 21 days at a light intensity of 1000 lux, relative humidity of 80%, and a temperature of 27.2° C. The experimental design was a random model. The measured growth parameters were greening of the cotyledon, hypocotyl length, and mean shoot length. Imbibition and radicle emergence was the measured time factors.

Percentage germination was assessed by using two methods: a) for salt shock by soaking the seeds in the respective concentration of saline solution for 48 hours in the dark; and b) by using filter paper and the Petri dish method at similar saline concentrations.

Declarations

Ethics Approval and Consent to Participate: Not applicable.

Consent for publication: Not applicable.

Data availability statement: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest: There is no conflict of interest.

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Author Contributions:

J Rachel Predeepa: Conceptualization, Investigation, Data Curation, Writing, Formal Analysis; **S Ranjith Kumar:** Conceptualization, Investigation, Data Curation, Formal Analysis; **G C Abraham:** Conceptualization, Resources; **T S Subramanian:** Resources, Supervision

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Abbreviations

IAA (Indole Acetic Acid), NaCl (sodium chloride), mM (millimolar), MS medium (Murashige and Skoog medium), % (percentage), C (Celsius), g l^{-1} (gram per litre), GA (gibberellic acid), 2,4-D (2,4-dichlorophenoxyacetic acid)

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Figures

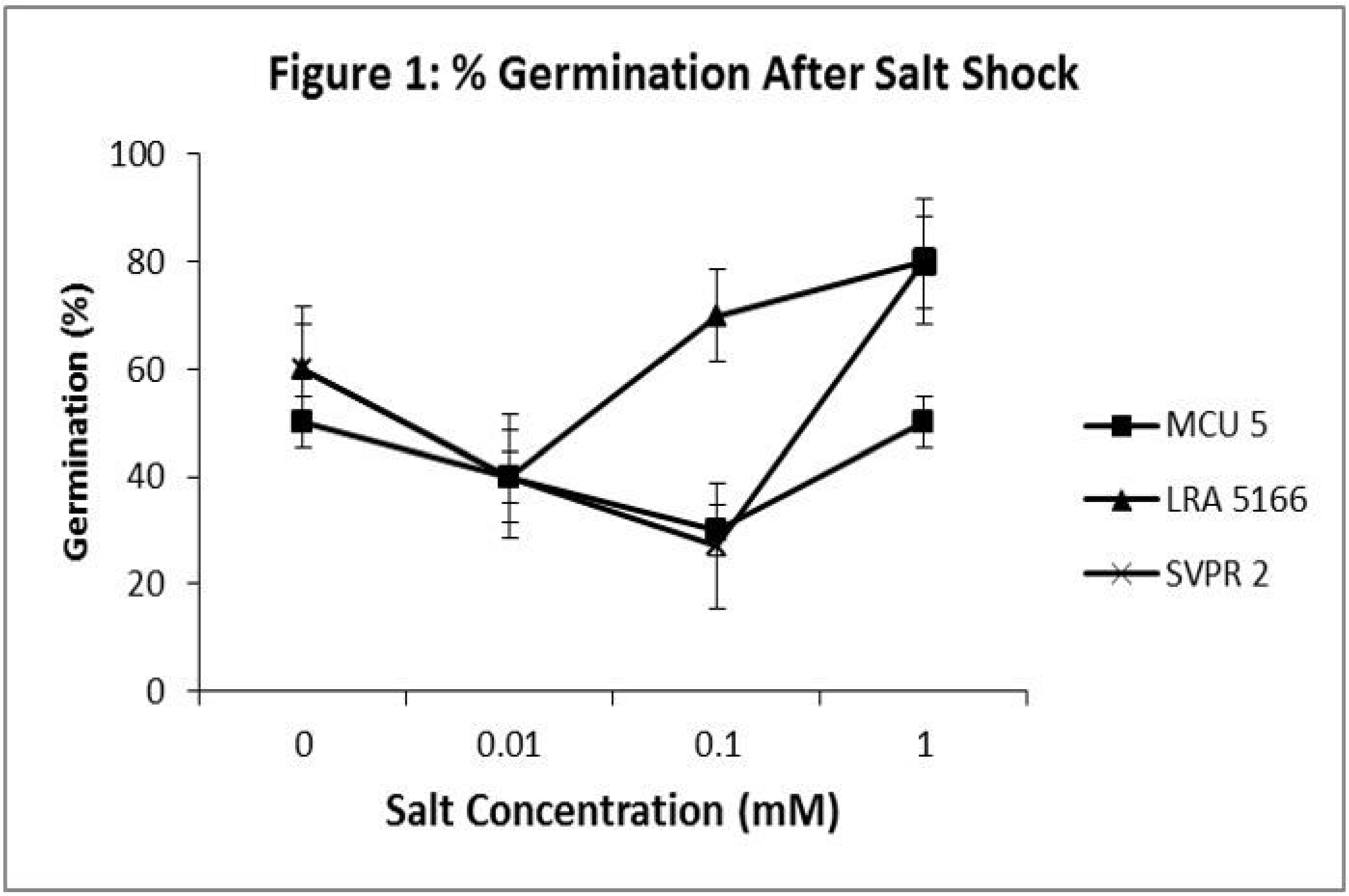

Figure 1

Percentage Germination of *Gossypium hirsutum* after Salt Shock

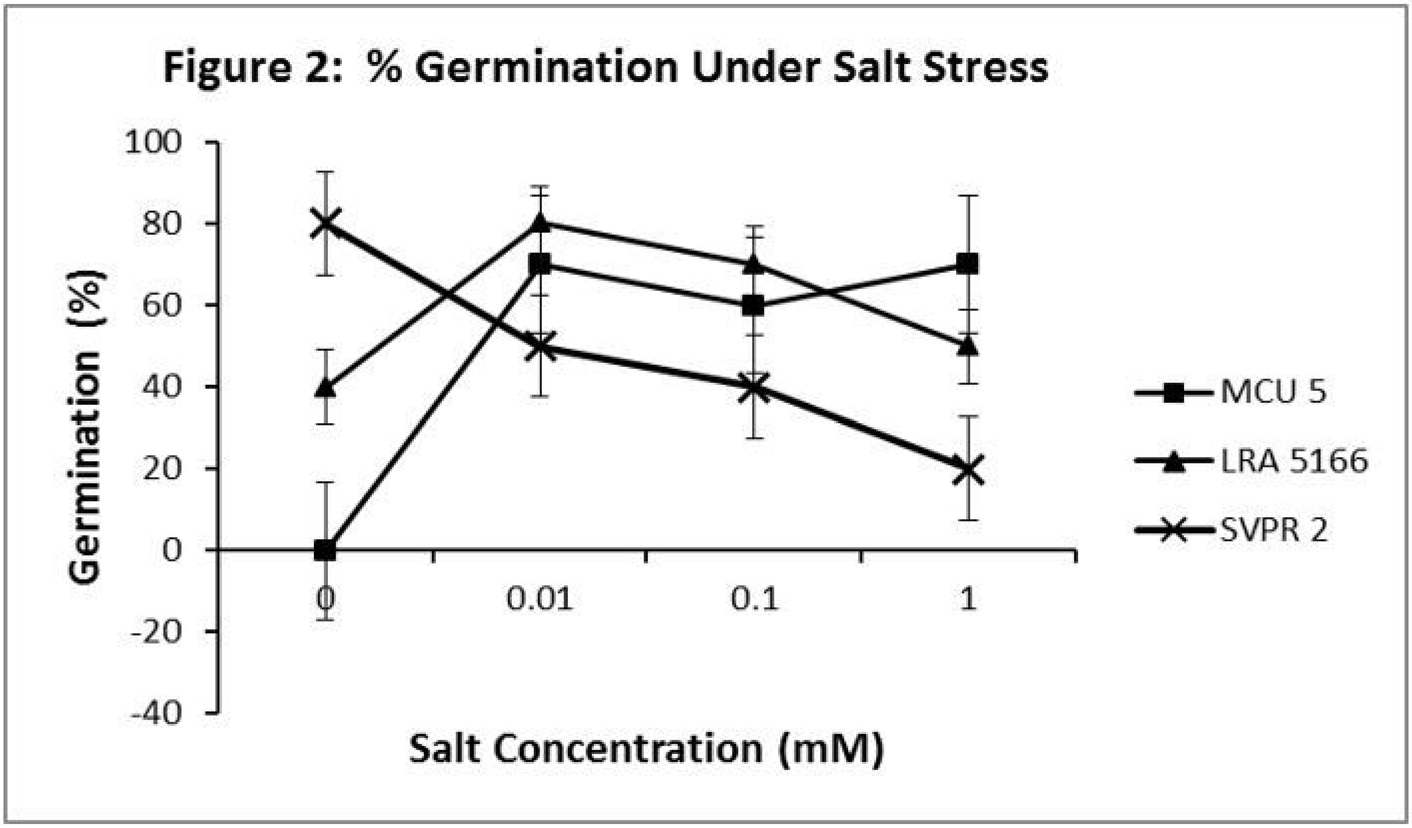
Figure 2: % Germination Under Salt Stress
100
80
60
40
20
0
-20
-40
Germination (%)
0
0.01
0.1
1
Salt Concentration (mM)
MCU 5
LRA 5166
SVPR 2

Figure 2