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Germination and early seedling growth of a medicinal plant giant milkweed (*Calotropis gigantea*) under salinity stress

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ABSTRACT

Giant milkweed (*Calotropis gigantea* L.) is a salinity and drought-resistant xerophyte that is widespread around the world and serves significant ecological and medicinal purposes. The research aimed to evaluate the influence of saline stress on germination characteristics and prompt growth attributes of seedlings of *C. gigantea*. Seeds were germinated under five salinity levels *viz*. 0, 6, 8, 10 and 12 dS m^{-1} and allowed to grow for 30 days for traits assessment. Germination percentage (GP), germination rate index (GRI), co-efficient of the velocity of germination (CVG), mean germination time (MGT), Timson germination index (TGI), shoot length (SL), root length (RL), seedling dry weight (SDW) and healthy seedling number at 30 days were found lower in the salt solution compared to the control condition. Mean germination time was expanded with the increment of salinity levels. TGI of *C. gigantea* sustained a significant positive linear regression with GP (r = 0.9881), GRI (r = 0.9923) and CVG (r = 0.7887) at $P < 0.001$, but MGT (r = 0.7855) at $P = 0.005$. The correlation coefficient among the germination traits revealed insignificant between RL and other germination traits (GP, CVG, MGT, and TGI) except GRI (r = 0.499*) and SL (r = 0.541*). It is recommended that, as an emerging medicinal and fiber resource plant, *C. gigantea* can be cultured productively in coastal saline areas.

Keywords: *Calotropis gigantea*, Seedling growth, Germination, Salinity stress, Medicinal plant

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Introduction

The genus *Calotropis* consists of two species viz. *Calotropis gigantea* and *Calotropis procera* belong to the Apocynaceae family (Islam *et al*., 2019). *C. procera* is well acquainted as the apple of sodom, rubber bush or rooster tree. It is called giant milkweed, crown flower, bowstring hemp or giant calotrope, which is locally called 'Aakanda' in Bangladesh. *C. gigantea* is native to Asian and tropical African nations of the world (Wang *et al*., 2008). It grows on fallow lands all over Bangladesh. *C. gigantea* is a rapidly rising, salt-tolerant or drought-resistant shrub that produces groups of white or violet waxy flowers (Sharma and Tripathi, 2009) and is recognized for its medicinal potential by scientists (Kumar *et al*., 2012). *Calotropis* is considered a multipurpose plant and both species of this genus have similar medicinal and pharmacological properties due to the presence of several essential organic compounds and secondary metabolites (Chan *et al*., 2016). In Bangladesh, China, Pakistan, and India, the species *C. gigantea* is widely used in conventional medicine (Parhira *et al*., 2016). The plant is used for the treatment of various influenzal, larvicidal, bacterial, fungal and cancer diseases (Sankar *et al*., 2014; Tariq *et al*., 2017). Both stem and seed fiber of *Calotropis* have commercial uses in making ropes, bowstrings, fishing nets, cushions and pillows (Kanchan and Atreya, 2016). Besides, the plant is also utilized for built-up natural fiber composites, which is an eye-catching substitute to artificial fiber in various uses (Muriira *et al*., 2018).

Negative impacts were observed on plant growth and development due to salt stress, which is responsible for decreasing yield (Hoque *et al*., 2023; Zheng *et al*., 2023). Salinity stress conveys water deficit stress that diminishes the osmotic potential of soil solutes, resulting in crop roots becoming unable to uptake water from the earth (Hannan *et al*., 2020) and the plant metabolic processes suffering due to toxicity (Imran *et al*., 2022). Salinity declined different germination traits such as germination proportion, length of root and coleoptile, callus mass, and growth attributes of seedling (Tarchoun *et al.*, 2022). Utmost numbers of plants are very perceptive to eminent salinity or ion stress during germination, emergence and first seedling growth (Alom *et al.*, 2016; Tobe *et al.*, 2005). Absorbing more Na^+ ions instead of K^+ results in worse production of plants in saline environments and might even be responsible for death (Alom *et al.*, 2016). Accumulation of proline in stresses (drought, salt and other environmental stresses) is the indicator of the tolerance mechanisms of plants (Yiu *et al.*, 2009). Germination and emergence are crucial stages for plant establishment in saline conditions. Salinity stress lessens the germination level and even hampers the initiation of the germination process (Hadjadj *et al.*, 2022). Salt tolerance is influenced by the seed germination and growth of the seedling (Malik *et al.*, 2022). Data, under various saline conditions are available on germination and early seedling growth of *C. procera* seeds (Al-Sobhi *et al.*, 2006; Galal *et al.*, 2015; Taghvaei *et al.*, 2012).

As far as we are aware, no investigation has been directed on the germination and seedling growth of *C. gigantea* seeds in a saline environment yet. To gain a deeper understanding of its biological and ecological characteristics, researchers wanted to explore its germination characteristics. Therefore, the present investigation brought new applied knowledge for cultivating the wild shrub *C. gigantea* in saline soil globally, especially in Bangladesh. Thus, this study was led to assess the germination as well as first seedling growth characteristics of *C. gigantea* under various levels of saline stress conditions.

Materials and Methods

Sample collection and preparation

The present investigation was directed at the Crop Physiology and Ecology laboratory of Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh (25°41´51.9´´ N, 88°39´17.1´´ E) during September and October 2020. The site elevation is 37.58 meters above sea level. Physiologically mature fruits of *C. gigantea* with open capsules and brown-coloured seeds were collected from fallow land at Nayanpur, Sadar in Dinajpur (25°39´12´´ N, 88°39´14´´ E). Ten pods were collected from three plants, and the distances among the plants were ≤ 2 m. Seeds were hand-extracted from the pods in July, mixed properly and placed to dry in the shade. The seeds were dried and stored in a freezer at a temperature of 5°C until sowing.

Experimental design and treatments

The experiment was designed using a completely randomized factorial method with seven levels of NaCl concentrations: 0, 6, 8, 10, 12, 14 and 16 dS m^{-1}. Experiments were completed with three replicates of 25 seeds each in Petri dishes (9 cm in diameter) filled with sand. Tap water was used as control. For preparing 1 dS m^{-1} solution, 640 mg NaCl/L or 0.64 g/L salt (NaCl) was used to add with 1 L tap water. So, per litre water 3.84, 5.12, 6.40, 7.68, 8.96 and 10.24 g NaCl were added to create solutions of 6, 8, 10, 12, 14 and 16 dS m^{-1}, respectively. Twenty-five seeds of *C. gigantea* were positioned in each Petri dish and rinsed every day with the required amount of control solutions, and kept at room temperature (25 ± 3°C).

Experimental data collection

The germination was calculated every 24 hours and the process was continued for 30 days. Seeds were considered germinated when the radicle was ≥ 1 mm long and visible (Bewley and Black, 1994). It was revealed that there were no or very few seeds germinated at 14 and 16 dS m^{-1} salinity levels. Hence, these two treatments were excluded during the recording and analysis of data in this experiment. Data was collected on various germination traits including germination percentage (GP), germination rate index (GRI), coefficient of the velocity of germination (CVG), mean germination time (MGT) and Timson germination index (TGI). Seedling growth was recorded at various plant growth stages. *C. gigantea* seedlings were grown for 30 days by daily addition of needed amount of solution. After 30 days of seed placement, five seedlings were randomly selected from each Petri dish and their growth traits were measured. Data on various growth traits for each treatment combination, *viz.* shoot length (SL), root length (RL), seedling dry weight (SDW) and healthy seedling number were also documented. The experimental data were collected using the following formulas:

- Germination percentage (GP) = (Germinated seeds/Total number of seeds) × 100 (El-Shaieny, 2015)
- Germination rate index (GRI) = $G_1/1 + G_2/2 + ... + G_i/i$ (Al-Mudaris, 1998)
  Where, G_1 = Germination percentage on day 1, G_2 = Germination percentage on day 2, and so on.
- Co-efficient of velocity of germination = $[100\ (V_1 + V_2 + \text{---------} + V_n)/V_1\ T_1 + V_2\ T_2 + \text{---------}\ V_nT_n]$ (Al-Mudaris, 1998)
  Where, V = Number of seeds germinated, T = Time (days) corresponding to V and n = Number of days to final count.
- Mean germination time (MGT) = $\Sigma\ Gx/\Sigma\ G$ (Al-Mudaris, 1998)
  Where, G represents the number of seeds that have germinated on day x.
- Timson germination index (TGI) = $\Sigma\ G/T$ (Ajmal Khan and Ungar, 1998)
  Where, G = Percentage of seed germinated per day, and T = Germination period.

Statistical analysis

All collected data were statistically analysed to find out the level of significance using the Statistix 10 program. Tukey's HSD Test was utilized to compare the mean differences at a significance level of 5%. Relative changes in stress condition over control for all studied parameters were measured using the formula: Relative change = [(Treatment - Control)/ Control] × 100. We also estimated correlation analyses (linear relationship between TGI and other germination traits and correlation coefficient among the germination and seedling growth attributes) among the studied traits.

Results

Effects of different salinity treatments on germination characteristics of Calotropis gigantea

Table 1 presents the results of germinating traits of *C. gigantea* seed under different salinity levels. Results showed that different salinity levels significantly affected the physiology of *C. gigantia* including GP, GRI, CVG, MGT, and TGI. The highest results were observed at control, followed by 6 dS m^{-1}, while the lowest results were found at 12 dS m^{-1} followed by 10 and 8 dS m^{-1}. The results exhibited that the control condition had the highest GP (98.67%) while increasing levels of salinity led to a decrease in GP (29.74-77.02%). The maximum GRI was found at control (99.64) followed by 6 dS m^{-1} (54.77), and the lowermost germination rate index was discovered at 12 dS m^{-1} (14.15), which was followed by 10 dS m^{-1} (28.02). The highest mean germination time (MGT) was detected at 12 dS m^{-1} (23.21 days), which was followed by 10 dS m^{-1} (22.02 days), and the lowest MGT was found at control (20.56 days). Under different salt stress, more decrease in Timson germination index (TGI) was found in 12 dS m^{-1} (10.49) followed by 10 dS m^{-1} (18.98). The intermediate (24.53) TGI was found at 8 dS m^{-1}. A reduction in TGI was observed in *C. gigantea* under different salinity levels. But the reduction was more prominent in 12 dS m^{-1} (10.49) as well as 10 dS m^{-1} (18.98) than in 8 dS m^{-1} (24.53) and 6 dS m^{-1} (36.71).

Table 1. Germination traits of *Calotropis gigantea* at 30 days after sowing under different levels of salinity.

Level of Salinity (dS m^{-1})	GP		GRI		CVG		MGT		TGI	
	%	RC (%)	Values	RC (%)	Values	RC (%)	days	RC (%)	Values	RC (%)
0	98.67^{a}	–	99.64^{a}	–	4.87^{a}	–	20.56^{c}	–	57.64^{a}	–
6	69.33^{b}	29.74	54.77^{b}	45.03	4.72^{ab}	-3.08	21.92^{b}	6.61	36.71^{b}	-36.31
8	42.67^{c}	56.75	38.34^{bc}	61.52	4.56^{bc}	-6.37	21.19^{bc}	3.06	24.53^{c}	-57.44
10	36.00^{c}	63.51	28.02^{cd}	71.89	4.54^{bc}	-6.78	22.02^{ab}	7.10	18.98^{cd}	-67.07
12	22.67^{d}	77.02	14.15^{d}	85.79	4.31^{c}	-11.49	23.21^{a}	12.89	10.49^{d}	-81.80
CV (%)	8.36	–	17.09	–	2.24	–	2.10	–	11.80	–

Mean followed by the similar letter (s) in the column was found insignificant at a 5% probability level under Tukey HSD. GP: Germination percentage; GRI: Germination rate index; CVG: Co-efficient of the velocity of germination; MGT: Mean germination time; TGI: Timson germination index; RC: Relative change over control

Effects of different salinity treatments on early seedling growth of Calotropis gigantea

The growth of 30-day-old seedlings was significantly influenced by the various NaCl concentrations (0, 6, 8, 10 and 12 dS m^{-1}) in sand culture. Findings revealed that various salt stresses have significant effects on all the growth parameters (SL, RL, SDW and HSN). With the increase in saline level, SL decreased. However, the magnitude of reduction was not the same for all salinity levels (Table 2). The highest shoot length (SL) of *C. gigantea* was found at the control condition (4.83 cm) followed by 6 dS m^{-1} (4.36 cm), and the lowest SL was found at 12 dS m^{-1} (2.41 cm) followed by 10 dS m^{-1} (3.48 cm). No statistical differences were found regarding root length (RL). However, when saline conditions increased, the RL decreased (Table 2). The highest seedling dry weight (SDW) was detected at control (0.0047 g), which was followed by 6 dS m^{-1} (0.0039 g), and the lowest seedling dry weight was found at 12 dS m^{-1} (0.0026 g), which was followed by 8 and 10 dS m^{-1} treatments (0.0032 and 0.0028 g, respectively). Results also showed that the several salinity levels had a considerable effect on the healthy seedling number of *C. gigantea* (Table 2). In the control condition, the highest (23.00) healthy seedling number was found, which was followed by 6 dS m^{-1} (16.00). Under different salt stress, a greater decrease in healthy seedling numbers was found in 12 dS m^{-1} (5.00).

Table 2. Different salinity (NaCl) effect on seedling growth of *Calotropis gigantea* at 30 days after sowing.

Level of Salinity (dS m⁻¹)	SL		RL		SDW		HSN	
	cm	RC (%)	cm	RC (%)	g seedling⁻¹	RC (%)	No.	RC (%)
0	4.83ᵃ	–	3.55	–	0.0047ᵃ	–	23.00ᵃ	–
6	4.36ᵃᵇ	-9.73	3.39	-4.51	0.0039ᵇ	-17.02	16.00ᵇ	-30.43
8	3.47ᵇᶜ	-28.16	2.87	-19.15	0.0032ᵇᶜ	-31.91	10.00ᶜ	-56.52
10	3.48ᵇᶜ	-27.95	2.99	-15.77	0.0028ᶜ	-40.43	8.00ᶜ	-65.22
12	2.41ᶜ	-50.10	2.82	-20.56	0.0026ᶜ	-44.68	5.00ᵈ	-78.26
CV (%)	13.06	–	19.31ᴺˢ	–	7.93	–	8.06	–

Mean followed by the same letter (s) in the column was found insignificant at a 5% probability level under Tukey HSD. SL: Shoot length; RL: Root length; SDW: Seedling dry weight; HSN: Healthy seedling number; RC: Relative change over control.

Correlation co-efficient among the germination and seedling growth attributes

The germination percentage of *C. gigantea* at 30 days after seeds sowing sustained a significant positive correlation with GRI (r = 0.967**) (Table 3). Correlation between MGT and other germination traits maintained a significant negative relation, whereas root length showed a non-significant negative correlation (r = -0.350NS). The association was found insignificant between RL and other germination traits (GP, CVG, MGT and TGI), whereas GRI and SL maintained a significant positive correlation with RL (r = 0.499*). Further, the highest significant positive correlation was found between TGI and GRI (r = 0.991**) at $P < 0.001$.

Table 3. The correlation coefficient among germination and seedling growth traits of *Calotropis gigantea* under saline solutions.

	GP	GRI	CVG	MGT	TGI	SL	RL	SDW
GP	–							
GRI	0.967**	–						
CVG	0.674**	0.795**	–					
MGT	-0.684**	-0.795**	-0.998**	–				
TGI	0.990**	0.991**	0.753**	-0.758**	–			
SL	0.883**	0.833**	0.592*	-0.611*	0.861**	–		
RL	0.475NS	0.499*	0.363NS	-0.350NS	0.483NS	0.541*	–	
SDW	0.948**	0.932**	0.653**	-0.660**	0.946**	0.869**	0.457NS	–

*GP: Germination percentage; GRI: Germination rate index; CVG: Co-efficient of the velocity of germination; MGT: Mean germination time; TGI: Timson germination index; SL: Shoot length; RL: Root length; SDW: Seedling dry weight; NS: Insignificant; **: Highly significant at 1%; *: Significant at 5%.*

Discussion

The seed germination stages and germinating characteristics are crucial to plants' developmental process. Under saline stress, a condition known as "germinating characteristics inhibition" frequently took place (Singha *et al.*, 2021). Establishing plants in saline soil is troublesome during seed germination and initial seedling stages (Al-Khateeb, 2006). Therefore five different salinity levels were considered (0, 6, 8, 10 and 12 dS m⁻¹) for analysis in present investigation. *C. gigantea* seeds were placed in the Petri dishes under 0, 6, 8, 10, 12, 14 and 16 dS m⁻¹ saline solutions. It was observed that no germination of seed took place at > 12 dS m⁻¹ saline solutions, and the highest germination was found in the control condition (Table 1). The results of our study were similar to a previous study (Galal *et al.*, 2015), where they observed the highest germination of *C. procera* was at no saline condition (98.70%), but germination at NaCl stress was 33.00%. Another research revealed that *C. procera* showed the highest germination percentage (99.00%) in distilled water, whereas the GP was 55.00% under saline conditions (Taghvaei *et al.*, 2012). This is due to imbibition being restricted by the solution's higher quantities of harmful ions and lower osmotic potential, which then in turn reduces germination (Wang *et al.*, 2020).

One study found (Galal *et al.*, 2015) that the MGT of *C. procera* in the control condition was 22.7 days, whereas in the saline condition, it was 24.20 days. A study (Tsegay and Gebreslassie,

2014) found the MGT of *Pisum sativum* seeds at 5 dS m^{-1} was higher than that of control. This means that as the salt concentration increases, the time taken for the seeds to germinate increases, indicating that the seeds take longer time to develop before they can begin to germinate and overcome the delay in germination time. Moreover, a previous study (Rajabi Dehnavi *et al.*, 2020) found that the MGT of genotypes of sorghum was 22.5 days at saline stress. An earlier investigation (Wu *et al.*, 2015) verified that compared to the control (distilled water), a saline solution of 10-200 mM meaningfully increased the MGT of sunflower. Our results further revealed that a higher level of NaCl restricted the germination of seeds and hindered the germination time in *C. gigantea*. Other crops such as rice (Xu *et al.*, 2011) and wheat (Akbarimoghaddam *et al.*, 2011) showed similar results. Rajabi Dehnavi *et al.* (2020) also found that the germination rate index of different genotypes of sorghum was 30.0-54.1 in various salinity stress conditions, which is similar to our present findings. Another study (Wu *et al.*, 2015) testified that a concentration of 200 mM salt caused a noteworthy decrease of GRI (14.80) in sunflowers (*Helianthus annuus* L.). These outputs are associated with the present findings. Due to the salt stress condition, the coefficient of germination was reduced in all salinity levels. A previous investigation (Panuccio *et al.*, 2014) found that with increasing seawater (saline water) concentration, the coefficient of the velocity of germination (CVG) decreased by 53% when exposed to 75% Seawater. Some investigations (Chakma *et al.*, 2019; Rofekuggaman *et al.*, 2020) revealed that increasing the levels of saline concentration decreased the seed's germination coefficient which is similar to our findings. A study (Wu *et al.*, 2015) found that a NaCl solution with a concentration of 10-200 mM significantly reduced the CVG of sunflowers when compared to the control (distilled water). These outputs are in parallel with the present results.

Several studies (Singha *et al.*, 2021; Hoque *et al.*, 2021) exhibited that the reduction of seedlings' growth due to salt stress disturbances the physiological parameters and subsequently causes several negative effects on the plant body. NaCl concentration inhibits plant development by osmotic stress and decreases plants' capacity to absorb water, which in turn inhibits growth (Shila *et al.*, 2016). When a plant receives an excessive quantity of salt, the NaCl concentration will ultimately increase to a critical level in aged transpiring leaves, producing premature ageing and reducing the plant's photosynthetic leaf number to a point where it can no longer support growth (Wang *et al.*, 2020). According to one study (Taghvaei *et al.*, 2012), the highest (4.9 cm) SL of *C. procera* in distilled water and the lowest (2.7 cm) SL was shown in the Saline stress condition. The SL of *C. procera* seedling at 500 mgL^{-1} saline condition was evidently lower (2.2 cm) than other saline treatments, and there were no discernible distinctions between them (Galal *et al.*, 2015). These results are in agreement with the present outputs (Table 2). One study (Taghvaei *et al.*, 2012) found that in the control condition, the RL of *C. procera* at 15 days seedlings was 1.5 cm, but at –0.05 Mpa, it was only 0.71 cm; after priming with salt and $CaCl_2$, RL increased (8.2 and 8.3 cm, respectively) but reduced as osmotic potential dropped. In addition, increasing saline concentrations significantly decreased the RL of *C. procera* seedlings (Galal *et al.*, 2015). According to a study (Xu *et al.*, 2011), the most prominent impact of NaCl was on roots; it may limit the attainment of seed germination as well as the establishment in their ecosystems. A previous study (Taghvaei *et al.*, 2012) found that the highest SDW of *C. procera* was shown in purified water, and the lowest SDW was recorded at -0.05 MPa. When irrigated with a saline solution exceeding 50 mM, the root dry weight, shoot dry weight, and overall dry weight of *Panicum turgidum* were significantly reduced ($P < 0.05$) (Al-Khateb, 2006). One study (Alom *et al.*, 2016) found that the SL, RL and SDW of twenty wheat varieties were higher at the control condition after 30 days of seed sowing compared to saline stress (4, 6, 8 and 10 dS m^{-1}). These outputs are associated with the present findings.

In *C. persica* (*C. procera*), all studied germination-related traits significantly correlated with the Timson germination index (TGI) at $P < 0.001$ (Al-Ansari and Ksiksi , 2016). These results are closely associated and support our findings (Table 3). However, TGI has been described as a superior exponent of seed germination rate (Al-Mudaris,1998). As TGI has been extensively and frequently used, the linear relations between TGI and each of the four other germination traits: GP, GRI, CVG, and MGT were assessed. The main purpose was to assess the agreement of each index with TGI, the most commonly used germination index (Al-Ansari and Ksiksi , 2016). After sowing *C. gigantea* seeds, the TGI at 30 days maintained a significant positive correlation with GP (r = 0.9881, $P < 0.001$), GRI (r = 0.9923, $P < 0.001$) and CVG (r = 0.7887, $P = 0.005$) but there was a significant negative correlation between mean germination time and TGI (r = 0.7855, $P = 0.005$). It was observed that MGT decreased with TGI (Fig. 1).

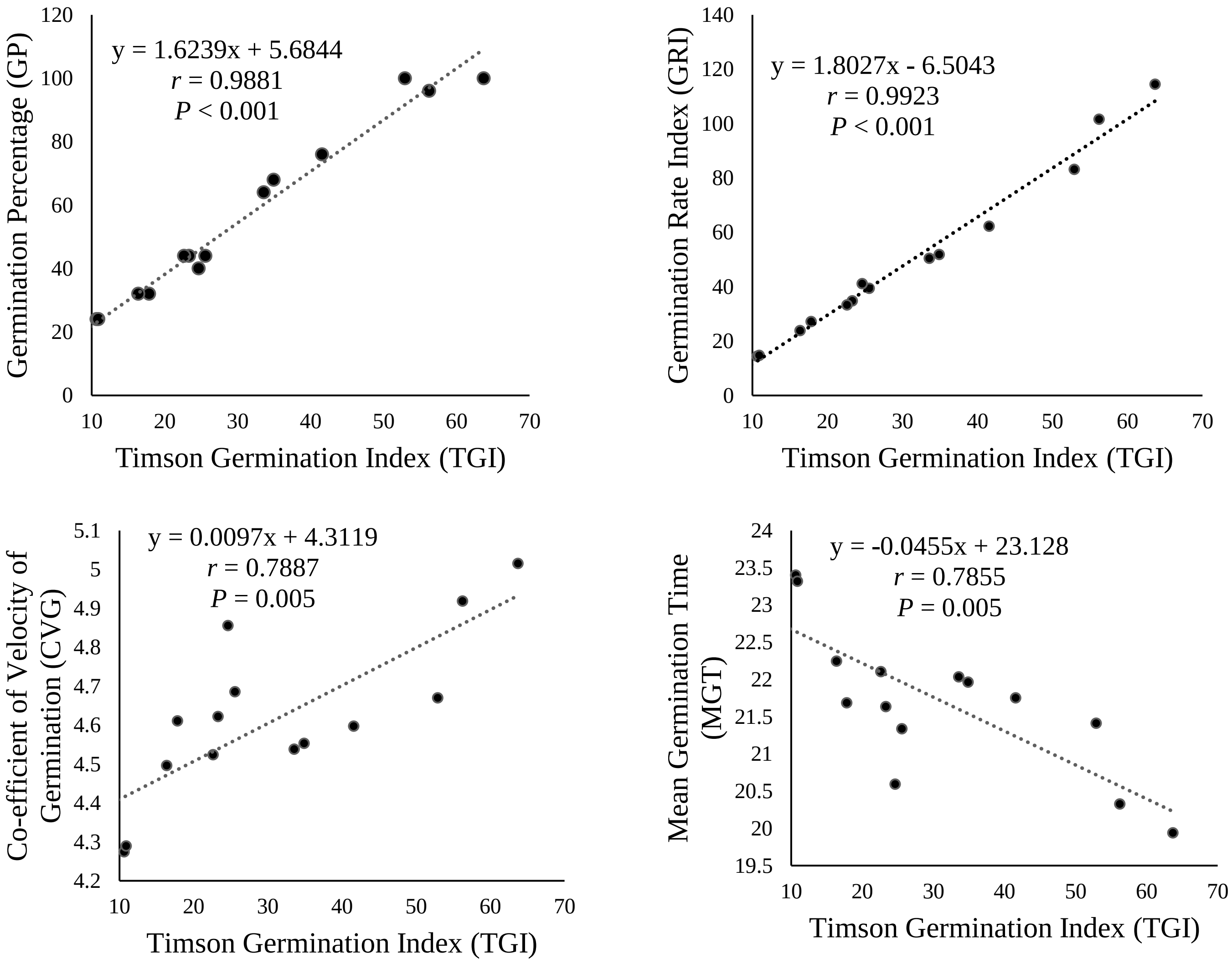

Fig. 1. Linear relationship between Timson germination index and other germination traits.

Conclusion

Under various forms and intensities of salinity stress, *C. gigantea* showed a species-specific response. In the present study, *C. gigantea* exhibited the lowest germination and early seedling growth at 12 dS m^{-1} and above the saline level the species showed no seed germination and feeble seedling growth. These results will assist in growing *C. gigantea*, a medicinal shrub that is a rich source of therapeutic components and may be utilized in a variety of treatments in a wide range of areas, especially for commercial cultivation of the Aakanda plant in the coastal saline regions of Bangladesh. In the future, it is recommended that the phenology, physiological traits and secondary metabolites of this plant be under saline conditions to understand their tolerance mechanism at the physiological and molecular level in Bangladesh.

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