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Original Research

Influence of Different Habitats' Seed and Their Sizes on Germination, Seedling Growth and Physiological Traits of a Medicinal Plant *Calotropis gigantea* L.

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Abstract

Habitat and seed size are the key factors that affect plant growth and development. The influence of these factors on the life history traits of the wild medicinal *Calotropis gigantea* is scanty around the globe. Hence, a laboratory and a pot experiment were conducted to investigate the impact of various sources (habitats) of seeds and their sizes on germination, seedling growth, and physiological traits of *C. gigantea* following a two-factor, completely randomized design with three repetitions. Factor-A comprised five habitats' seeds (T_1-roadside, T_2-railway line, T_3-river bank, T_4-waste dump, and T_5-grazing land), and factor-B consisted of three sizes (mass) of seeds (L-large, M-medium, and S-small). In this study, we observed that the grazing land habitats' seed (T_5) performed better than other sources of seeds for various studied traits. The performances of different sizes of seeds were categorized as L > M > S. Overall, it was notable that the greater performances of different habitats' seeds with larger sizes were characterized as $T_5 \times L > T_1 \times L > T_2 \times L > T_3 \times L > T_4 \times L$ for germination, seedling growth, and physiological attributes. The present findings might have provided greater insights into the growth, development, and productivity of *C. gigantea* useful to farmers, practitioners, and researchers.

Keywords: *Calotropis gigantea*, habitat, seed size, physiological traits, seedling growth

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Introduction

The genus *Calotropis* is a perennial shrub comprised of two species (*C. procera* and *C. gigantea*) that are distributed in tropical and subtropical regions of Africa and Asia. The species *Calotropis gigantea* L. is common in Bangladesh, Burma, India, China, Pakistan, Malaysia, Indonesia, the Philippines, Sri Lanka, and Thailand [1]. It is a restorative herb mostly known as giant milkweed and locally known as "Akando" in Bangladesh. It is a xerophyte that can resist salinity and drought stress conditions, has substantial ecological importance in conserving biodiversity, and has remedial values for various diseases [2]. Researchers examined the plant and found it exhibits insecticidal, antibacterial, antifungal, antipyretic, anti-cancerous, analgesic, cytotoxic, and wound-healing activity [3-6]. Furthermore, most rural people depend on it as traditional medicine [7]. Muriira et al. [8] reported that *Calotropis* has significant economic potential for the textile industry due to the quality of fibers obtained from seeds. Because of cost-effectiveness, easy accessibility, reliability, and a broad range of applications, scientists have given considerable attention to the production of this plant. The plant grows naturally up to 900 meters in elevation along open habitats, i.e., roadsides, railway embankments, riverbanks, wasteland, and grazing lands, and can withstand a wide range of climatic changes and blooms throughout the year [5]. Presently, it is considered an endangered and medicinally red-listed species in some regions of the globe due to overexploitation [1, 9, 10].

Seed culture is an easy and widely used method for the commercial propagation of various medicinal plant species [11]. In many plants, propagation and seedling growth are affected by the natural growing habitats (i.e., maternal raising sites) where seeds are produced, in addition to the size (seed mass) of those seeds [12, 13]. Changes in habitat quality are considered the most crucial factors affecting the viability of the plant populations and their persistence, which lead to effective germination, vigor, and growth of the plants over time [14]. The maternal habitat under certain environmental conditions affects plant fitness, germination, seed dormancy, and seedling growth [15, 16]. For instance, herbs, shrubs (*Anabasis setifera*, *Zygophyllum coccineum*), carex, and alpine plants lead different germination and seedling growth mechanisms depending on their several habitats' origins, *i.e.,* cool, warm, wetland, dry-site, forest, and open habitat [17, 18]. In many species, it has been observed that the germination, emergence, growth of the seedlings, and physiological traits are severely affected by some plant characteristics (seed mass, seed coat, nutrient and moisture content of seeds, etc.) and environmental factors (habitat, temperature, light, relative humidity, pH, salinity, drought, etc.) [19, 20]. Furthermore, seed size has been reported as inclusive of ecological consequences across various species of plants with regard to germination, emergence, seedling growth, establishment, and yield [21-24]. Researchers have found that larger-sized seeds sprout faster or show higher germination percentages [23, 25]. Steiner et al. [26] examined *Arachis hypogaea* and found that large-sized seeds produce more vigorous seedlings and accumulate greater dry matter. However, Vidak et al. [24] reported that small-sized seeds of *Phaseolus vulgaris* have rapid emergence and the highest variation in the emergence period.

Several other studies have exhibited that the physiological traits of plants are also affected by habitat and seed size [18, 27]. Nonetheless, the relationship patterns between seed mass and seedling growth traits are not considered universal, apparently varying with environmental, ecological, and evolutionary conditions. In the current study, our fervent intention was to justify whether the smaller, medium, or large-sized seeds of *C. gigantea* are suitable for their optimal germination, growth, and development. The seeds of *C. gigantea* are dispersed mainly through wind and fall on diverse habitats of Bangladesh, such as roadsides, railway lines, riverbanks, waste dumps, and grazing land. The environmental and soil conditions of these habitats are not similar. For this reason, the ecological fitness, survival capacity, and aftermath development of *C. gigantea* seedlings do not proceed at the same pace in various habitats. Therefore, we evaluated for the first time the influence of various habitats' seeds and their sizes on germination, emergence, seedling growth, and physiological attributes of *C. gigantea.* This study might be helpful to different stakeholders (e.g., researchers, farmers, traditional practitioners, etc.) for optimum production of medicinal inputs from giant milkweed.

Materials and Methods

Seed Collection

Mature seeds of *C. gigantea* were obtained from five natural habitats, e.g., roadside, railway line, riverbank, waste dump, and grazing land of Dinajpur district, Bangladesh (Table 1), during July 2022. After collection, the seeds were carefully dried under ambient conditions at 23±2ºC for 7 days and categorized as small, medium, and large using an average seed mass of 5.46 mg, 6.38 mg, and 7.70 mg seed^{-1}, respectively, following the method of Kołodziejek [28]. Finally, the seeds were stored in the refrigerator at 4ºC till the experimental set-up.

Experimental Site, Design, and Treatments

The studies were carried out at the lab and research field of Hajee Mohammad Danesh Science and Technology University, Bangladesh (25°41′51.9″ N and 88°39′17.1″ E). To assess the germination traits of giant milkweed, a lab experiment was conducted from September to October 2022 in Petri dishes

Table 1. Seed sampling locations (habitats) with elevation and average seed mass of *Calotropis gigantea.*

Habitat	Longitude (E)	Latitude (N)	Elevation (m)	Average seed weight (mg)		
				Large Size	Medium Size	Small Size
Roadside	88°44′22.6306″	25°46′23.16756″	45.0	8.10	7.06	5.98
Railway line	88°36′48.6579″	25°37′16.92804″	36.0	7.86	6.06	5.00
River bank	88°38′11.1930″	25°39′15.35004″	34.0	7.38	6.98	5.62
Waste dump	88°39′00.2412″	25°38′4.94844″	40.0	6.94	5.12	4.60
Grazing land	88°39′14.2167″	25°39′11.33964″	36.0	8.22	6.70	6.12
Mean	-	-	-	7.70	6.38	5.46

(diameter: 11 cm and depth: 1.8 cm) filled with sand under normal ambient conditions (average room temperature and relative humidity were 25±2ºC and 80±10%, respectively). The pot assay was conducted in the research field using a plastic pot filled with soil (pH = 5.25, organic carbon = 0.97%, organic matter = 1.67%, available N = 0.084%, available P = 42.68 µg/g, and exchangeable K = 0.33 meq/100g) during October to January 2022 under normal environmental conditions (average temperature, relative humidity, and rainfall were 20.63ºC, 87.83%, and 94.40 mm, respectively, during the growing period) for evaluation of emergence, seedling growth, and physiological traits of *C. gigantea*. Before seed sowing, organic matter (5%) and triple super phosphate (2.5 g) were properly mixed in each pot as a foundation. After 30 days of seed sowing, potassium chloride (10 g) and urea (10 g) were applied in each pot to enhance growth and development. Both experiments were conducted following a two-factor completely randomized design (CRD) with three replications, where factor A consisted of five habitats' seeds (T_1 = Roadside, T_2 = Railway line, T_3 = River bank, T_4 = Waste dump, and T_5 = Grazing land), and factor B comprised three sizes (mass) of seed (L = Large, M = Medium, and S = Small).

Assessment of Germination and Early Seedling Growth Traits

Seeds were surface sterilized in 1% mercuric chloride solution for 5 minutes to prevent bacterial and fungal attacks. Then, the seeds were rinsed with distilled water, and twenty-five seeds were placed in 12 cm diameter and 1 cm high Petri dishes filled with sand. The sand was sieved to remove debris and stones before use. Each assay contained 75 seeds, with 3 repetitions of 25 seeds per Petri dish. All Petri dishes were well watered by this period. According to Bewley and Black [29], seeds were considered germinated when the radicle length was about ≥1 mm. The germination percentage (GP, %), mean germination time (MGT, days), germination rate index (GRI), co-efficient of velocity of germination (CVG), and Timson's germination index (TGI) were calculated 15 days after sowing. Then, only five seedlings were allowed to grow for 30 days to obtain the early seedling growth parameters (root length, shoot length, and seedling dry weight). The germination traits were calculated using the following Equations (1-5):

$$GP = \sum Ng / Nt \quad (1)$$

Where Ng = number of germinated seeds and Nt = total number of seeds [30].

$$MGT = \sum (Ni \times Ti) / \sum Ni \quad (2)$$

$$GRI = \sum (Ni / i) \quad (3)$$

$$CVG = \{\sum Ni / \sum (Ni \times Ti)\} \times 100 \quad (4)$$

Where Ni = number of seeds germinated on day i, and Ti = number of days from sowing [31, 32].

$$TGI = \sum Gt / T \quad (5)$$

Where Gt = percentage of seed germination per day and T = germination period [33].

Evaluation of Emergence and Seedling Growth Parameters

In a pot assay, twenty seeds were sown in a 10 L plastic pot (27 cm × 25 cm × 18 cm). At 20 days after seed placement, the seedling emergence traits were estimated. Then, only five healthy seedlings were allowed to grow, and at 40 days, the growth attributes were examined. The seedling emergence percentage (SEP), emergence index (EI), and mean emergence time (MET) were calculated using the following Equations (6-8):

$$SEP = (\text{Total number of seedlings emerged} / \text{Total number of seeds sown}) \times 100 \quad (6)$$

$$EI = \sum (Et / Dt) \quad (7)$$

Where Et = number of emerged seeds, and Dt = counted days [34].

$$MET = \sum Dn / \sum n \quad (8)$$

Where D = Number of days counted from the beginning of emergence, n = Number of seeds that emerged on day D [35].

Seedling growth traits, such as the number of leaves in plant^{-1}, were counted manually, shoot and root length were measured by the scale, and the seedlings were dried at 70ºC for 72 hours in an electric oven to determine the seedling dry weight. The total leaf area was determined by the following formula (Equation 9):

$$\text{Leaf area} = W \times L \times 0.75 \quad (9)$$

Where W = width of leaf (cm), L = length of leaf (cm), and 0.75 is a constant [36].

Table 2. Germination traits of *Calotropis gigantea* influenced by habitats' seeds and their sizes at 15 days after sowing.

Treatment		GP (%)	MGT (days)	GRI	CVG	TGI
Habitat						
T_1		97.33 a	5.63 ab	80.21 a	18.93 bc	53.49 ab
T_2		90.13 b	4.72 bc	62.40 ab	22.06 ab	45.65 bc
T_3		95.72 ab	4.42 c	84.52 a	23.79 a	56.23 a
T_4		71.20 c	4.13 c	51.02 b	25.18 a	37.48 c
T_5		92.00 ab	6.41 a	79.31 a	16.22 c	53.60 ab
P-value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Seed Size						
L		95.36 a	5.60 a	83.13 a	23.24 a	55.20 a
M		92.48 a	5.07 ab	73.29 ab	21.34 ab	49.63 a
S		80.33 b	4.51 b	58.06 b	19.13 b	43.05 b
P-value		< 0.001	0.003	0.002	0.005	< 0.001
Habitat × Seed Size						
T_1	L	99.20 a	6.69	94.48	22.77	59.98 a
	M	99.20 a	5.80	91.49	18.28	56.52 ab
	S	93.60 a	4.40	52.68	15.73	44.19 ab
T_2	L	90.40 ab	4.85	69.64	22.45	47.38 ab
	M	100.00 a	4.80	61.56	22.29	45.73 ab
	S	80.00 bc	4.49	56.01	21.45	43.86 ab
T_3	L	99.20 a	5.40	93.57	26.74	59.98 a
	M	96.00 a	4.07	74.11	25.45	55.20 ab
	S	92.00 ab	3.80	85.88	19.19	53.54 ab
T_4	L	97.60 a	4.24	77.08	25.86	54.64 ab
	M	73.60 c	4.17	45.41	25.15	35.88 bc
	S	42.40 d	3.99	30.58	24.57	21.94 c
T_5	L	90.40 ab	6.85	80.89	18.37	54.25 ab
	M	93.60 a	6.52	80.12	15.55	54.82 ab
	S	92.00 ab	5.87	76.91	14.76	51.74 ab
P-value		< 0.001	0.519	0.416	0.561	0.032
CV%		6.30	21.62	33.65	20.00	19.41

Here, GP = germination percentage, MGT = mean germination time, GRI = germination rate index, CVG = coefficient velocity of germination, TGI = Timson's germination index, T_1 = roadside, T_2 = railway line, T_3 = river bank, T_4 = waste dump, T_5 = grazing land, L = large, M = medium, S = small size of seed.

Physiological Attributes

After evaluating seedling growth traits, plants were thinned out at 40 days, and only one seedling was allowed to grow for up to 60 days for further physiological evaluations. Physiological attributes such as various photosynthetic pigments (chlorophyll and carotenoids) and proline content were estimated using fresh leaf tissues of 60-day-old plants. Photosynthetic pigments were assessed according to the methods proposed by Lichtenthaler [37] using the formula below:

$$Chl_a = 11.244A_{661.6} - 2.04\,A_{644.8}$$

$$Chl_b = 20.13A_{644.8} - 4.19A_{661.6}$$

$$Chl_{a+b} = 7.05A_{661.6} + 18.09A_{644.8}$$

$$Car_{x+c} = (1000A_{470} - 1.90C_a - 63.14C_b) / 214$$

Here, Chl_a = $Chlorophyll_a$; Chl_b = $Chlorophyll_b$; Chl_{a+b} = Total Chlorophyll; Car_{x+c} = Total Carotenoids. A standard curve was used to determine the proline concentration, which was calculated on a fresh weight basis using the formula established by Bates et al. [38]. µmoles proline / g of fresh weight material = {(µg proline / ml × ml toluene) / 115.5 µg / µmole} / {(g sample)/5}.

Data Analysis

All results data were analyzed using the program Statistix 10 [39]. A two-way ANOVA and Tukey's honestly significant difference (HSD) test at the $P \leq 0.05$ level were applied as a post hoc test to evaluate significant differences between the means of the treatments.

Results

Germination and Early Seedling Growth Traits

Table 2 presents the germination and early seedling growth traits of *C. gigantea*. It was observed that the combined effects of various habitats' seeds and their sizes on germination percentage and Timson's germination index were significantly varied, but mean germination time, germination rate index, and co-efficient germination velocity were insignificant. The highest GP was observed in railway line habitat with medium-sized seed (T_2 × M, 100%), followed by T_1 × L, T_1 × M, and T_3 × L treatments (99.20%), while the lowest germination percentage was found in T_4 × S (42.40%). The highest TGI (59.98) was observed in T_1 × L and T_3 × L, while the lowest TGI (21.94) was found in T_4 × S. Besides, the T_3, T_4 and T_5 habitats' seeds showed the highest GRI (84.52), CVG (25.18), and MGT (6.41 days), respectively, while the large size seeds exhibited the highest results over small and medium size seeds for these traits.

Data presented in Table 3 indicate the combined and significant effects of various habitats' seeds and their sizes on early growth traits such as shoot length, root length, and seedling dry weight. The highest shoot length (SL) was found in T_3 × L (5.50 cm), followed by T_2× L (4.99 cm) and T_5× L (4.94 cm) treatments, whereas the lowest SL was found in T_2 × S (3.73 cm). The highest root length (RL) was observed in T_2 × L (3.86 cm), while the lowest RL was noticed in T_4 × S (2.30 cm). The highest seedling dry weight (SDW) was observed in T_2 × L (0.045 g) and followed by T_2× L and T_5 × L (0.043 g), but the lowest SDW was examined in T_3 × S (0.021 g), which is followed by T_5 × M (0.024 g) and T_1 × S (0.025 g) treatments.

The correlation coefficient (*r*) between the germination and early growth traits of *C. gigantea* is presented in Table 4. The results revealed that most of the traits showed significant correlations. The strong positive correlation observed in TGI-GRI (r = 0.9359***) is followed by TGI-GP (r = 0.6996***), GRI-GP (r = 0.5375***), SDW-SL (r = 0.4755***), SDW-RL (r = 0.4494***), GRI-MGT (r = 0.4421***), TGI-MGT (r = 0.3912***), RL-GP (r = 0.3821***), and RL-SL (r = 0.3602***), while the lowest significant correlation was found in SL-GP (r = 0.2432*). A negative significant correlation was observed among some traits, *viz.,* CVG-MGT, GRI-CVG, and TGI-CVG. An insignificant positive correlation was observed between GP-SDW, MGT-RL, MGT-SDW, CVG-SL, CVG-RL, and CVG-SDW. In addition, insignificant negative correlations were found between CVG-GP, GRI-SDW, and TGI-SDW.

Emergence and Seedling Growth Traits

Table 5 shows the significant combined effect of different habitats' seeds and their sizes on seedling emergence percentage but insignificant for the emergence index and mean emergence time. The highest SEP was observed in roadside habitats with large-size seeds (T_1 × L, 98.66%), which was statistically similar to T_1 × M, T_2 × L, and T_2 × M (98.00, 98.33, and 97.00%, respectively); however, the lowest SEP was found in T_4 × S (55.00%). Besides, the highest EI and MET were found in plants in the T_1 and T_4 habitats, respectively. Moreover, the large-size seeds showed the highest SEP and EI (84.00% and 20.70, respectively).

The results showed that the combined effects of habitats' seeds and their sizes on the number of leaves plant^{-1} (NLPP), shoot length (SL), root length (RL), seedling dry matter (SDM), and leaf area (LA) were significant (Table 6). The maximum NLPP was observed in T_5 × L (11.17) and followed by T_3 × L (9.95), whereas the minimum NLPP was found in the T_4 × S (6.95) combination followed by T_4 × M (7.00). The highest SL was observed in the T_3 × L treatment (19.20 cm), which was followed by T_5 × L (18.00 cm), while the lowest SL

Table 3. Early seedling growth traits of *Calotropis gigantea* influenced by habitats' seeds and its sizes at 30 days after sowing.

Treatment		SL (cm)	RL (cm)	SDW (g)
Habitat				
T_1		4.29	3.12 a	0.029 b
T_2		4.46	3.28 a	0.042 a
T_3		4.55	2.88 ab	0.028 b
T_4		4.48	2.51 b	0.032 b
T_5		4.53	2.51 b	0.029 b
P-value		0.325	<0.001	<0.001
Seed Size				
L		5.02 a	3.21 a	0.039 a
M		4.40 b	2.98 a	0.031 b
S		3.97 c	2.40 b	0.025 c
P-value		< 0.001	< 0.001	< 0.001
Habitat × Seed Size				
T_1	L	4.85 abc	3.47 abc	0.036 a-d
	M	4.13 cde	3.22 a-d	0.027 d-g
	S	3.89 de	2.70 cd	0.025 efg
T_2	L	4.99 ab	3.86 a	0.045 a
	M	4.67 bcd	3.68 ab	0.043 ab
	S	3.73 e	2.70 cd	0.039 abc
T_3	L	5.50 a	3.43 abc	0.035 bcd
	M	4.26 b-e	2.84 bcd	0.030 c-f
	S	3.88 de	2.40 d	0.021 fg
T_4	L	4.81 abc	2.64 cd	0.036 a-d
	M	4.49 b-e	2.60 cd	0.035 b-e
	S	4.16 cde	2.30 d	0.025 efg
T_5	L	4.94 abc	2.66 cd	0.043 ab
	M	4.48 b-e	2.60 cd	0.024 fg
	S	4.17 cde	2.30 d	0.036 g
P-value		0.027	0.025	< 0.001
CV%		8.20	14.51	13.49

Here, SL = shoot length, RL = root length, SDW = seedling dry weight, T_1 = roadside, T_2 = railway line, T_3 = river bank, T_4 = waste dump, T_5 = grazing land habitat, L = large, M = medium, S = small size of seed

was found in the T_4 × S (10.50 cm), followed by T_1 × S (12.68 cm). The highest RL was found in the T_5 × L treatment (18.33 cm), whereas the lowest RL was found in T_4 × S (8.72 cm), followed by T_4 × M (9.21 cm), T_2 × S (10.83 cm), T_3 × S (10.85 cm), and T_1 × S (11.66 cm). Moreover, the highest SDM was found in T_5 × L (0.816 g), whereas the lowest SDM was shown in T_4 × S (0.230 g), followed by T_4 × M (0.237 g). The highest LA was observed in T_3 × S (29.11 cm^2), followed by T_5 × L (27.62 cm^2), while the lowest LA was found in T_2 × S (14.82 cm^2), followed by T_1 × M (15.93 cm^2) and T_3 × L (16.23 cm^2) treatments.

Physiological Attributes

In Table 7, the combined effects of habitats' seeds and their sizes on $chlorophyll_a$ and proline contents were significant, but $chlorophyll_b$, total chlorophyll, and total carotenoids were insignificant. The highest Chl_a was observed in T_5 × L (14.40 mg g^{-1} FW), followed by T_4 × L

Table 4. Correlation coefficient (*r*) among the studied traits of *Calotropis gigantea.*

Trait	GP	MGT	CVG	GRI	TGI	SL	RL	SDW
GP	-							
MGT	0.2737*	-						
CVG	-0.1783^{NS}	-0.4096***	-					
GRI	0.5375***	0.4421***	-0.2958**	-				
TGI	0.6996***	0.3912***	-0.3205***	0.9359***	-			
SL	0.2432*	0.2778*	0.2097^{NS}	0.3115**	0.2853**	-		
RL	0.3821***	0.1020^{NS}	0.2097^{NS}	0.2474*	0.2266*	0.3602***	-	
SDW	0.1409^{NS}	0.0404^{NS}	0.2097^{NS}	-0.0445^{NS}	-0.0259^{NS}	0.4755***	0.4494***	-

Here, SL = shoot Length, RL = root Length, SDW = seedling dry weight, GP = germination percentage, MGT = mean germination time, CVG = co-efficient velocity of germination, GRI = germination rate index, TGI = Timson's germination Index, *, **, and *** indicate correlation co-efficient (*r*) is significant at P≤0.05, P≤0.01, and P≤0.001, respectively; NS = non-significant.

Table 5. Seedling emergence traits influenced by habitats' seeds and their sizes of *Calotropis gigantea* at 20 days after sowing.

Treatment		SEP (%)	EI	MET (days)
Habitat				
T_1		94.00 a	23.46 a	20.47 c
T_2		92.11 a	21.09 b	21.43 c
T_3		74.11 c	15.81 d	23.94 b
T_4		59.66 d	12.77 e	24.83 a
T_5		76.77 b	17.42 c	23.43 c
P-value		< 0.001	< 0.001	< 0.001
Seed Size				
L		84.00 a	20.70 a	21.91 c
M		81.66 b	17.90 b	22.70 b
S		72.33 c	15.73 c	23.86 a
P-value		< 0.001	<0.001	<0.001
Habitat × Seed Size				
T_1	L	98.66 a	24.94	19.99
	M	98.00 a	24.43	20.43
	S	85.33 b	21.00	21.00
T_2	L	98.33 a	23.31	20.87
	M	97.00 a	21.63	21.43
	S	81.00 bc	18.33	22.00
T_3	L	77.66 cd	19.31	22.73
	M	76.00 de	14.30	23.77
	S	68.66 fg	18.83	25.33
T_4	L	64.00 gh	15.31	23.73
	M	60.00 h	12.50	24.77
	S	55.00 i	10.50	26.00

T_5	L	81.33 bc	20.64	22.21
	M	77.33 cd	16.63	23.10
	S	71.66 ef	15.00	25.00
P-value		< 0.001	0.081	0.619
CV%		2.04	6.25	3.72

Here, SEP = seedling emergence percentage, EI = emergence index, MET = mean emergence time, T_1 = roadside, T_2 = railway line, T_3 = river bank, T_4 = waste dump, T_5 = grazing land habitat, L = large, M = medium, S = small size of seed.

Table 6. Seedling growth traits of *Calotropis gigantea* as influenced by habitats' seeds and their sizes at 40 days after sowing.

Treatment		NLPP	SL (cm)	RL (cm)	SDM (g plant^{-1})	LA (cm^2)
Habitat						
T_1		8.25 b	16.12 ab	12.03 bc	0.369 b	17.69
T_2		8.20 b	15.16 b	12.35 b	0.459 ab	18.01
T_3		9.07 a	16.63 a	12.55 b	0.441 b	22.21
T_4		7.32 c	12.58 c	10.81 c	0.380 b	17.50
T_5		9.16 a	16.80 a	15.01 a	0.601 a	22.55
P-value		< 0.001	< 0.001	< 0.001	< 0.001	0.008
Seed size						
L		9.26 a	17.01 a	14.61 a	0.589 a	19.97
M		8.39 b	16.12 b	12.22 b	0.400 b	19.13
S		7.56 c	13.25 c	10.81 c	0.360 b	19.68
P-value		< 0.001	< 0.001	<0.001	<0.001	0.819
Habitat × Seed size						
T_1	L	8.35 c-f	17.83 ab	12.25 bcd	0.440 bcd	18.89 abc
	M	8.66 b-e	17.83 ab	12.17 bcd	0.381 bcd	15.93 c
	S	7.75 def	12.68 ef	11.66 b-e	0.285 cd	18.27 abc
T_2	L	8.83 bcd	16.25 bcd	13.68 bc	0.565 abc	18.08 abc
	M	8.00 c-f	15.33 b-e	12.53 bc	0.407 bcd	21.13 abc
	S	7.78 def	13.89 de	10.83 cde	0.405 bcd	14.82 c
T_3	L	9.95 ab	19.20 a	14.29 b	0.455 bcd	16.23 c
	M	9.27 bc	16.26 bcd	12.50 bc	0.432 bcd	21.29 abc
	S	8.00 c-f	14.43 de	10.85 cde	0.437 bcd	29.11 a
T_4	L	8.00 c-f	13.75 de	14.50 b	0.673 ab	19.03 abc
	M	7.00 f	13.50 de	9.21 de	0.237 d	16.97 bc
	S	6.95 f	10.50 f	8.72 e	0.230 d	16.51 bc
T_5	L	11.17 a	18.00 ab	18.33 a	0.816 a	27.62 ab
	M	9.00 bcd	17.66 abc	14.70 b	0.543 a-d	20.32 abc
	S	7.33 ef	14.75 cde	12.00 bcd	0.445 bcd	19.69 abc
P-value		< 0.001	0.028	< 0.001	0.028	0.002
CV%		5.79	6.25	8.22	24.05	18.85

Here, NLPP = number of leaves plant^{-1}, SL = shoot Length, RL = root Length, SDM = seedling dry matter, LA = leaf area, T_1 = roadside, T_2 = railway line, T_3 = river bank, T_4 = waste dump, T_5 = grazing land habitat, L = large, M = medium, S = small size of seed.

Table 7. Photosynthetic pigments and proline content of leaves at 60 days after sowing.

Treatment		mg g^{-1} FW				Proline (μmole g^{-1} FW)
		Chl_a	Chl_b	Chl_{a+b}	Car_{x+c}	
Habitat						
T_1		10.84 b	3.31 c	13.93 bc	3.84 ab	2.96 ab
T_2		10.70 b	3.40 bc	13.83 c	3.75 b	2.70 bc
T_3		10.93 b	3.38 bc	14.18 abc	3.87 ab	1.91 d
T_4		12.58 a	4.00 ab	16.73 ab	4.51 ab	2.67 c
T_5		12.68 a	4.21 a	16.85 a	4.58 a	3.10 a
P-value		< 0.001	0.008	0.004	0.007	< 0.001
Seed Size						
L		12.81 a	3.55 b	16.68 a	4.47 a	2.57 b
M		11.12 b	4.01 a	14.68 b	4.20 a	3.09 a
S		10.60 c	3.41 b	13.95 b	3.65 b	2.35 c
P-value		<0.001	0.006	0.004	0.002	< 0.001
Habitat × Seed Size						
T_1	L	12.29 bc	3.21	15.49	4.28	3.01 bc
	M	10.36 fg	3.40	13.76	3.77	3.25 ab
	S	9.85 g	3.33	12.54	3.46	2.61 cde
T_2	L	11.40 cde	3.59	15.16	3.96	2.74 b-e
	M	10.81 def	3.40	13.21	4.14	2.92 bcd
	S	9.89 fg	3.21	13.10	3.14	2.43 cde
T_3	L	12.05 bc	3.69	15.75	4.44	1.79 fg
	M	10.56 efg	3.27	13.50	4.10	2.19 efg
	S	10.19 fg	3.17	13.31	3.07	1.74 g
T_4	L	13.89 a	4.15	18.10	4.63	2.39 c-f
	M	12.47 b	4.19	16.67	4.57	3.25 ab
	S	11.40 cde	3.65	15.43	4.32	2.38 def
T_5	L	14.40 a	3.13	18.92	5.05	2.90 bcd
	M	11.98 bc	5.79	16.28	4.43	3.84 a
	S	11.65 bcd	3.70	15.36	4.26	2.56 cde
P-value		0.002	0.068	0.999	0.727	0.011
CV%		2.68	13.25	13.86	13.88	7.72

Here, Chl_a = chlorophyll $_a$, Chl_b= chlorophyll $_b$, Chl_{a+b} = total chlorophylls, Car_{x+c} = total carotenoids, T_1 = roadside, T_2 = railway line, T_3 = river bank, T_4 = waste dump, T_5 = grazing land habitat, L = large, M = medium, S = small size of seed.

(13.89 mg g^{-1} FW), whereas the lowest Chl_a was found in T_1 × S (9.85 mg g^{-1} FW). In terms of proline content, the highest was estimated in T_5 × M (3.84 µmole g^{-1} FW), whereas the lowest was found in the T_3 × S (1.74 µmole g^{-1} FW) combination. Besides, the highest total chlorophyll, total carotenoids, and proline contents were found in grazing land (T_5) habitats' plants (16.85 mg g^{-1} FW, 4.58 mg g^{-1} FW, and 3.10 µmole g^{-1} FW, respectively). Moreover, the large-size seeded plants exhibited the highest total chlorophyll (16.68 mg g^{-1} FW) and carotenoid (4.47 mg g^{-1} FW) contents.

Discussion of Results

Germination and early seedling growth are the most sensitive stages in the life cycle of a plant and are controlled by different factors [40]. Several environmental factors (e.g., maternal habitat, light, temperature, relative humidity, latitude, altitude, etc.) and plant traits (such as seed size, seed coat, etc.) manipulate the germination, early seedling growth, and development of temperate herbs, shrubs, and legumes in both laboratory and field conditions [19, 20, 41]. Among these, seed size greatly affects the germination percentage and dormancy of the seeds, where larger seeds show a higher germination rate, followed by medium and small seeds [42-44]. The habitats correlate with the germination of seeds and morphological traits of plants [18, 45]. El-Keblawy et al. [46] mentioned the germination of seeds from different habitat types has been linked to the influence of genetic and environmental factors. A similar investigation confirmed that seeds collected from calcareous and siliceous habitats showed a variance in germination percentage under different temperature conditions [18]. In the current study, we observed that roadside habitat × large-size seed (T_1 × L) gave significantly the highest GP and TGI, which was statistically similar to grazing land habitat × large-size seed (T_5 × L). Besides, the other germination traits (MGT, GRI, and CVG) were insignificant among the habitat × seed size treatments. Large-sized seeds were always superior to medium- and small-sized seeds regarding various germination traits. Wang et al. [47] reported that larger seeds had the highest GP and survival percentage compared to the medium- and small-sized seeds of *Anabasis aphylla.* Similarly, Domic et al. [44] found that the heavy seeds provided almost twofold GP than the medium-sized and ninefold GP than the small-sized seeds in *Polylepis tomentella*; however, the survival rate was similar in large and medium-sized seeds. Petersen [25] observed that the large-sized seeds of *Pinus elliottii* exhibited the highest CVG, MGT, and GRI, whereas the lowest was observed in small-sized seeds in control conditions. These results support the present findings. We found that the larger seeds revealed the higher GP, GRI, CVG, and TGI. This may occur due to having more food material in reserved conditions, which positively supported the germination process.

In the case of early seedling growth, we observed that larger-sized seeds from various habitats produced a higher shoot length, root length, and seedling dry weight (Table 3). The highest shoot length and sturdiness were produced in *Pinus elliottii* when large seeds were used, whereas the lowest was observed with small-sized seeds [25]. Tenikecier and Genctan [22] investigated wheat and found that variation in seed sizes had a significant effect on the yield-contributing characters, yield, and quality of grain, where the decreased seed size exhibited inferior performance in most of the traits. The correlation coefficient results among the study traits indicated a significant positive correlation in GP-RL, GP-SL, MGT-TGI, SL-SDW, and RL-SDW. According to the previous study, positive correlations between GP-RL and negative correlations among GP-SL were found in *Sesbania* species seedling growth that was influenced by seed mass [48]. Islam et al. [49] mentioned the significant negative correlation in TGI-MGT that contrasts our results. Further, Li et al. [50] examined the morphological traits of *Saussurea salsa* in three different habitats and observed that seeds sown in different environmental conditions significantly varied in specific root length, root shoot ratio, and other seedling traits. These findings support our outputs.

Lamichhane et al. [51] reported that environmental factors have a crucial effect on seedling emergence, ultimately affecting seedling growth and development. In the present investigation, large-size seeds showed the highest significant results regarding seedling emergence and growth traits in terms of SEP, EI, NLPP, SL, RL, and SDW compared to the small-size seeds (Tables 5 and 6). Oyewole and Patience [23] revealed that small, medium, and large-sized seeds of *Arachis hypogea* had significant effects on seedling emergence time, flowering time, weight of 100 seeds, and length of the tap root, where the large-sized seeds revealed the lowest emergence time. The effects of seed size and color were studied in the common bean (*Phaseolus vulgaris* L.). It was found that seed size had significant effects on traits such as mean germination time (MGT) and emergence index (EI), while the color of the seeds did not have a significant effect [52]. Similar to our findings, it was reported that large-seeded plants increased the leaf number per plant and enhanced SL, RL, and SDW compared to the small-seeded plants in the *Solanum tuberosum* [21] and *Arachis hypogaea* [26] species. Again, a strongly positive correlation among different sizes of seeds and shapes with the leaf area, dry mass of leaf, and distinctive leaf area was found in maize plants [53]. We observed that the roadside habitats' seeds showed the highest results in terms of SEP and EI. It could be because the roadside habitats' seeds have a well-established and effective adaptive performance compared to the other habitats' seeds. Each habitat has some challenges, but certain plants acclimatize themselves to thrive in specific conditions. Combinedly, from several habitats' seeds and their sizes, the grazing land habitats with large-size seeds revealed the highest significant results in terms of NLPP, RL, and SDW (Table 6). This is because the open sunlight conditions probably support the plants in accumulating maximum photosynthates in grazing land. This environmental condition triggers large seed mass production and facilitates higher seed germination, emergence, and life history traits [14]. Velázquez-Rosas et al. [54] investigated the larger seeds having more food reserves and producing more vigorous seedlings with higher seedling dry mass and root-shoot ratio than smaller seeds.

Chlorophyll, carotenoid, and proline have been widely studied physiological traits that have been influenced by plant and environmental factors like maternal seed size, light, temperature, etc. Tudela-Isanta et al. [18] examined that the variation of habitats affects the physiological performance of seedlings. Díaz-Pérez and John [55] reported that the shade environment influences plant growth, leaf color, and chlorophyll in *Capsicum annum*. The plants' biomass and seedling survival were correlated with the seed size and the available light gradient present in the environment [56]. In the current study, grazing land habitats and the large-sized seeds individually showed the highest results in terms of total chlorophyll, total carotenoid, and proline contents. This may be due to the open field conditions in the grazing land, which allow the plants to capture more sunlight. Large-sized seeds reveal vigorous germination, and they provide stronger seedlings that can withstand adverse environmental conditions [14]. This ultimately yields more photosynthetic pigments, which is similar to our findings. Moreover, the large-seeded *Calotropis* plants showed the highest significant results in terms of total chlorophyll and carotenoid contents. Besides, the variation of habitats notably affects the water potential of the leaf, morphological and anatomical traits, and photosynthetic parameters [27]. Plants grown in extreme environmental conditions produce higher proline levels to alleviate the ill effects of stressful situations [57-59]. The medium-sized seeds of *Calotropis* from grazing lands, waste dumps, and roadside habitats exhibited higher proline contents (Table 7) because, in these sites, the maternal plants from which the seeds were collected survived under harsh environmental conditions [59, 60]. On the other hand, plants from riverbank habitats' seeds produced lower levels of proline compared to the other habitats' seeds. This is because riverbank plants might get the required amount of water for their growth and development and face less stressful conditions than the other studied habitats. Hopefully, this experiment will provide key information regarding germination, emergence, seedling growth, and physiological traits that are influenced by various natural habitats' seeds and their sizes for ecological adaptation of the medicinal *Calotropis gigantea* plant.

Conclusions

In conclusion, the study indicated that the sizes of seeds of *C. gigantea* collected from different habitats have substantial variations regarding their germination and growth traits. Our results revealed that seeds from grazing land habitats with large size ($T_5 \times L$) significantly accomplished greater performance than the other treatment combinations. However, further investigations are necessary to strengthen in-depth knowledge on the germination requirements of *C. gigantea*, which seems to be sensitive to its habitats' environmental conditions and the sizes of the seeds.

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Conflict of Interest

There is no conflict of interest in the article.

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