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RESEARCH

Impacts of invasive weed *Chromolaena odorata* on growth and development of threatened native tree *Aegle marmelos* under water stress conditions

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Background: The invasive weed *Chromolaena odorata* has been pronounced in dry habitats in Nepal, where a threatened native tree *Aegle marmelos* prefers to grow. It is known that the allelochemicals from *C. odorata* can harm native species differently, however, the impact may depend on environmental factors such as soil moisture in the invaded habitats. This study evaluates how the native tree responds to leaf and root products (leachates) of *C. odorata* and how water stress influences this interaction. For evaluating these facts, field survey to know the *A. marmelos* seedling status in *C. odorata* invaded and uninvaded sites was carried out. The seedlings were also grown in pots by treating the pot soil with *C. odorata* root and leaf leachates under water-stressed and unstressed conditions. Morphological and biochemical traits (root and shoot length, seedling biomass, leaf and root number, specific leaf area (SLA), photosynthetic pigments and proline) were measured.

Results: A significant decrease in *A. marmelos* seedling population was found within its habitat under the canopy of *C. odorata*. Root length and biomass of *A. marmelos* seedlings were decreased by water stress (frequent drought). Addition of *C. odorata* leaf and root leachates further increased the toxic effects. Similarly, the content of photosynthetic pigments (chlorophylls and carotenoids) was decreased under drought conditions, with *C. odorata* leachates contributing more to the reduction in chlorophyll amount. The increment of the osmolyte proline in the native seedlings shows the seedlings are experiencing stressful environment.

Conclusions: Seedlings of *A. marmelos* are facing a stressful environment in their habitat invaded by *C. odorata*. Frequent dryness and exposure of the native seedlings to invasive leachates together can significantly decline the population of threatened native species. Hence, removal of *C. odorata* from the invaded habitat is recommended before the onset of monsoon season to prevent leaching from its aerial or underground parts to minimize the negative impacts on the growth and development of such a threatened native tree.

Keywords: invasions, leachate toxicity, near threatened species, seedling regeneration

Introduction

Chromolaena odorata (L.) R.M. King & H. Rob. of the family Asteraceae, native to the neotropical region, has been one of the aggressively spreading invasive weeds (Zachariades et al. 2009). It has been categorized as one of the 100 worst invasive plant species in the world (Lowe et al. 2000). Its invasion poses a significant threat to various ecosystems in subtropical to tropical regions (Koutika and Rainey 2010). Commonly, it prefers to invade roadsides, fallow areas, and forest ecosystems forming dense monocultures (Thapa et al. 2016; Tiébré and Gnanazan 2018). In invaded habitats, the weed displaces native species, alters species composition, severe negative impacts on forage for livestock and crops (Shackleton et al. 2017; Thapa et al. 2016). Such detrimental effects of *C. odorata* on native ecosystems have become a serious environmental challenge.

Besides the overall impacts of *C. odorata*, the studies particularly focused on the impact of *C. odorata* on native tree species have described the plant as a harmful weed in forest ecosystems due to having its adverse impacts on seedling and sapling populations of tree species (Gbètoho et al. 2018; Shackleton et al. 2017; Thapa et al. 2016). Specific characteristics of the weed including rapid growth and development, formation of monoculture stands and dense canopy, competition for resources, and allelopathy (Chandrasekaran and Swamy 2010; Hu and Zhang 2013; Mandal and Joshi 2014; Zheng and Liao 2017) are considered as the mechanisms behind aforementioned negative influences of the weed. Also, alteration in soil nutrients and soil microbial attributes according to the needs of its establishment are indirect mechanisms to harm native plants in invaded areas (Debnath et al. 2018; Koné et al. 2021; Nirmala et al. 2023).

The distribution range of *C. odorata* is widening spatially and temporally with land use and land cover changes and anthropogenic disturbances (Adhikari et al. 2023; Akin-Fajiye and Akomolafe 2021). Also, it has shown adaptability in response to climate change scenarios and future projections suggest that the spread of the weed will be significantly influenced by climate-related factors (Sharma et al. 2022a; Thiney et al. 2019). In Southeast Asia, including Nepal, *C. odorata* has extended its distribution beyond fallow lands, roadsides, and degraded areas to the tropical to subtropical forests (Joshi et al. 2006; Sharma et al. 2022b). Particularly, its colonization is extreme in low-lying Tarai regions and also reaches up to the Siwalik regions and mid-hills in central and western Nepal (Dhakal et al. 2018; Thapa et al. 2016).

Importantly, tropical and subtropical regions in Nepal are characterized by the presence of the highly valuable native tree *Aegle marmelos* (L.) Corrêa, belonging to the family Rutaceae. The plant has great medicinal value with edible fruits and its wood is utilized as timber (Pathirana et al. 2020; Waheed et al. 2023). The tree is an important element of mixed forests and it is classified as the "Near Threatened" category on the International Union for Conservation of Nature (IUCN) Red List (Baral and Upreti 2016; Plummer 2020). The areas of *A. marmelos* in certain regions like the Ramechhap district of Nepal have been experiencing prolonged periods of low precipitation (Dahal et al. 2016; Poudel and Chettri 2021). Unfortunately, the same habitats have been heavily invaded by *C. odorata* for about a decade.

In such a scenario, *A. marmelos* might be facing two kinds of stresses: 1) the water stress in the area with low precipitation and 2) the invasions of *C. odorata*. In response to these stresses, the growing seedlings of plants may undergo modifications in their morphology (root, shoot, and leaves), physiology, and biochemistry (Ahmad et al. 2018). The increasing severity and frequency of drought worldwide and in Nepal may be associated with intensification *C. odorata* invasion (Dahal et al. 2016; Damberg and AghaKouchak 2014; Sharma et al. 2022a). Given the conditions, it was hypothesized that the concurrent stress of both water scarcity and *C. odorata* invasion have more detrimental effects on the growth and development of *A. marmelos* compared to the effects of water stress alone. Executing experiments to test this hypothesis is crucial to shed light on potential correlations among drought, invasions, and response of native species like *A. marmelos*. Such a study is essential for understanding and addressing the impact of environmental stressors on native biodiversity and ecosystems.

Materials and Methods

Study area and seedling survey

The study area of the field assessment was the Ramite Community Forest in the Manthali Municipality of Ramechhap district within the Bagmati Province, Nepal (27.3814°N to 27.3843°N and 86.0417°E to 86.0409°E, with an elevation ranging 469–630 m above sea level [a.s.l.]) (Fig. 1). This area is characterized by a combination of riverine and mixed forests as the Tamakoshi river, one of the major tributaries of the Koshi river, flows nearby the area.

The average annual rainfall and temperature at the site are recorded as 222.6 mm per year and 18.9°C, respectively (Climate Data 2023). *Aegle marmelos* is one of the dominant native trees in the forest including other trees such as *Senegalia catechu* and *Annona squamosa* as associated trees. Shrubs like *Rhus parviflora* and *Cipadessa baccifera* and grasses like *Saccharum spontaneum* and *Arundinella nepalensis* are also common in the forest. Field observation revealed the persistent invasion of *C. odorata* throughout the entire forest area (Fig. S1A).

A survey for seedling assessment of *A. marmelos* in the community forest was conducted in June 2022. It involved the sampling of a total of 18 square plots, each of size 5 × 5 m^2, to assess the density of *A. marmelos* seedlings (Fig. S1B). Horizontal transects were made within the forest to sample the plots at a distance of 200 m apart. Besides counting seedlings, the cover of *C. odorata* in each plot was also estimated visually (5%, 10%, 15%, 25%, and so on) considering each plot as 100%.

Pot experiment

After the field survey, pot experiments were conducted to analyze the effect of *C. odorata* stresses on the growth and development of *A. marmelos* (Fig. S1C). Seeds of *A. marmelos* were collected from mature fruits of a tree within the community forest (27.3984°N, 86.0604°E, 547 m a.s.l.). The seeds were then transported to the Central Department of Botany, Tribhuvan University, Kirtipur, Nepal. They were dried and stored in the refrigerator at 4°C until use. The seeds were placed onto moist filter paper and allowed to germinate in the dark at room temperature (25°C ±

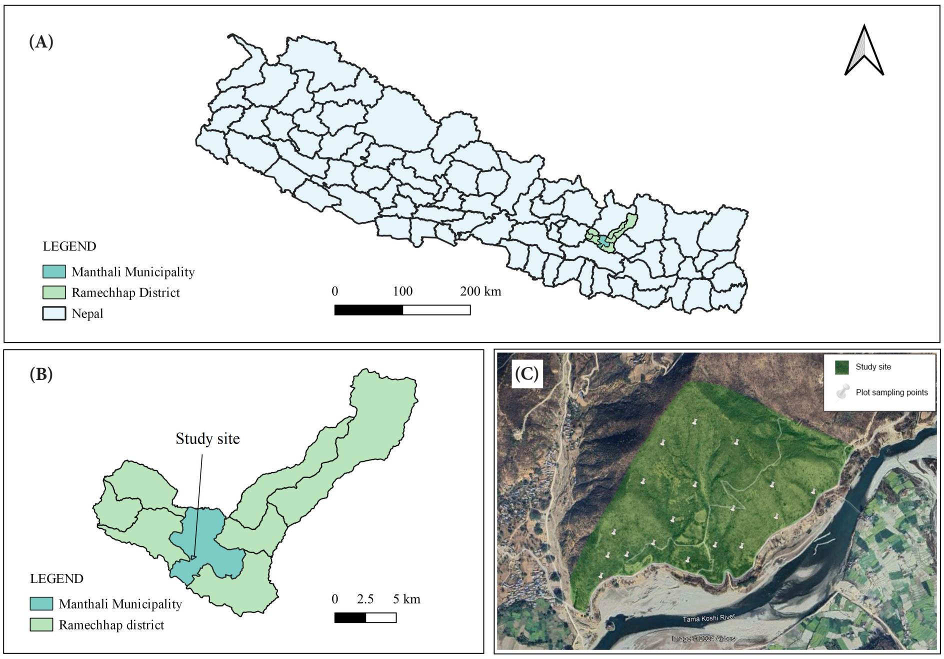

Fig. 1 Map showing study site. (A) Nepal showing Ramechhap district. (B) Ramechhap district showing Manthali Municipality and study site. (C) Distribution of sampling plots in study site (source: Google Earth).

5°C). The seeds germinated and after a week, the radicle length of seedlings reached up to 0.5 to 1.5 cm.

Polyethylene pots of size 13 cm in height and 11 cm in width were filled with a mixture of 800 g of garden soil and sand in a ratio of 2:1 (soil: sand). The seedlings of uniform length (1.5 cm) were transplanted into the pots. Three sets of pots were prepared for the following treatments:

1) Water: designated as ‘water’ treatment

2) Leachate of *C. odorata* leaves: designated as ‘leaf leachate’ treatment

3) Leachate of *C. odorata* roots: designated as ‘root leachate’ treatment.

For the preparation of leaf and root leachates, fresh leaves and roots of *C. odorata* were collected from Nepalthok, Bagmati Province, Nepal (27.443°N and 85.818°E; 550 to 600 m a.s.l.). Leaves and roots were soaked in distilled water (DW) (10 g leaves or roots /100 mL DW, separately) for 24 hours to obtain the respective leachates. It was the simulation that mimicked the natural washing away of the leachate from *C. odorata* leaves and the diffusion of exudates from roots into the soil in the invaded sites. It is difficult to predict the exact concentration of leachate that has been released in nature. High amount of leaves or roots may release high concentration of allelochemicals, which may have very strong negative effect on seedlings, and in nature, such situation may occur during infrequent rainfall events. Conversely, the leaves < 10 g may release of less concentration of allelochemicals, which is unlikely to have significant effect on seedling growth. This scenario may occur during regular rainfall. To address both extremes, we expected that *C. odorata* in its dense stand would release leachate with concentration equivalent to the 10 g fresh leaves per 100 mL of water.

The pots of treatments as mentioned above were further exposed to (a) regular irrigation by water or leachate (control) and (b) irrigation was done only after the seedlings wilt (it was intermittent irrigation and considered as the drought). Fifty milliliters of water and leachates were added in an alternate day on respective treatment pots for regular irrigation until the harvesting time of seedlings. Conversely, for the drought treatment, the same volume of water or leachate was provided after wilting signs in the leaves of seedlings. The seedlings with drooping leaflets were considered as the wilted seedlings.

These treatments were simulated as regular rainfall and intermittent rainfall in nature. In Nepal, the monsoon season has been marked by frequent rainfall, which keeps the soil moist. However, recent trends indicate that there is shifting towards irregular and less frequent rainfall even during monsoon season, especially in our study area (Poudel and Chettri 2021). This shifting has resulted frequent droughts, which can create stress on plants due to low soil moisture during growing season. Our study design mimics the above mentioned situations i.e., regular irrigation mimics frequent rainfall and intermittent irrigation mimics less frequent rainfall, exposing seedlings to drought condition (low soil moisture). We have used *C. odorata* leachates as well, which means there is leaching of substances from its aerial parts during either frequent or less frequent rainfall.

Overall, there were following conditions: 1) regular irrigation with water (control), 2) intermittent irrigation with water (seedlings facing drought intermittently, low moisture), 3) regular irrigation with *C. odorata* leaf leachate, 4) intermittent irrigation with the leaf leachate (seedlings facing drought with intermittent exposure to leaf leachate), 5) regular irrigation with *C. odorata* root leachate, 6) intermittent irrigation with the root leachate (seedlings facing drought with intermittent exposure to root leachate). Soil moisture in the drought treatment pots was 10%–20% during the time of seedling wilting, while the moisture in the regularly irrigated pots was maintained 60%–70% throughout the experiment.

Each of the treatments had 9 replicated pots and each pot contained a single seedling of *A. marmelos*. The seedlings in the pots were grown in a greenhouse. The temperature in the greenhouse during the experiment ranged from 17°C to 30°C, relative humidity between 70% to 85%, and daylight intensity ranging from 5,000 to 6,000 Lux. The position of the pots was changed every alternate day to minimize the positional effect.

Measuring functional traits of seedlings

Seedlings of *A. marmelos* were harvested after 97 days after seedling transplantation (Fig. S2). During harvesting, the plants' morphological and biochemical traits were evaluated. Shoot and root length were measured and the number of leaves and secondary roots were counted. Specific leaf area (SLA) was calculated by dividing the leaf area by the leaf dry mass (Vile et al. 2005). Biomass of oven-dried shoot and root (80°C for 72 hours) was also measured.

The biochemical parameters, proline, and photosynthetic pigments (chlorophyll and carotenoids) were estimated as these parameters frequently fluctuate when plants are exposed to environmental stresses. Leaves were sampled from the seedlings in each pot for analyzing biochemical traits. Photosynthetic pigments (chlorophyll *a* and *b*, total chlorophylls, and carotenoids) concentrations were estimated following Bajracharya (1999). The sampled leaves (500 mg) from each replicated pot were ground with 50 mL of 80% acetone to extract the pigments. Then the absorbance was measured using spectrophotometer at 440 nm, 645 nm and 663 nm, respectively. The pigment concentration was calculated using following formulae:

$$\text{Chlorophyll } a \ (\text{mg.l}^{-1}) = 9.78 \times A_{663} - 0.99 \times A_{645}$$

$$\text{Chlorophyll } b \ (\text{mg.l}^{-1}) = 21.4 \times A_{645} - 4.65 \times A_{663}$$

$$\text{Carotenoids } (\text{mg.l}^{-1}) = 4.69 \times A_{440} - 0.268 \, (20.2 \times A_{645} + 8.02 \times A_{663})$$

$$\text{Total chlorophyll } (\text{mg.l}^{-1}) = \text{Chlorophyll } a + \text{Chlorophyll } b$$

Similarly, the proline concentration was measured in the leaves of seedlings following the method established by Bates et al. (1973). Leaf samples (0.25 g) were macerated with 5 mL of sulphosalicylic acid (3%) and centrifuged for 7 minutes (5,000 rpm). Thereafter, 2 mL of the supernatant was carefully pipetted and transferred to another test tube, where it was combined with an additional 2 mL of glacial acetic acid and 2 mL of acid-ninhydrin. This mixture was then incubated in a hot water bath for 1 hour, followed by ice bath to arrest the reaction. Subsequently, 4 mL of toluene was added, and the upper layer (chromophore) was carefully pipetted and the absorbance value was recorded at 520 nm using spectrophotometer. The concentration of proline was estimated from standard curve and calculated using the formula:

$$[(\mu\text{g proline/mL} \times \text{mL toluene}) \ / \ 115.5 \ \mu\text{g}/\mu\text{mole}] \ / \ [(\text{g sample})/5] = \mu\text{moles proline/g of fresh weight material.}$$

Data analysis

A linear regression was performed to analyze the relationship between the number of *A. marmelos* seedlings and *C. odorata* cover. One-way analysis of variance (ANOVA) was used to compare the differences in functional traits of *A. marmelos* seedlings among the stress conditions (water, leaf leachate, and root leachate) except for count data. Poisson regression was used to compare the variation among the treatments for the number of leaves and secondary roots. Independent sample t-test was employed to analyze the differences in functional traits of seedlings between regular and intermittent irrigations. Statistical analyses were performed using the software R (version 4.3.1).

Results

Effect of *C. odorata* cover on *A. marmelos* seedlings

The number of seedlings of *A. marmelos* were counted within each quadrat sampled in the field. The number of seedlings per quadrat decreased with an increase in the cover of *C. odorata*. The analysis showed a significant negative relationship between the seedling density of *A. marmelos* (number/quadrat) and *C. odorata* cover ($p < 0.001$, $r = -0.82$). The cover of *C. odorata* ranged from 15% to 80% (Fig. 2).

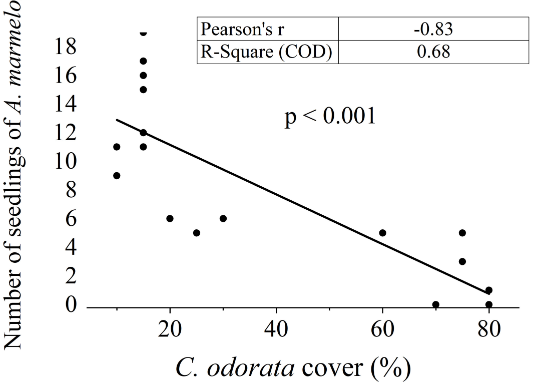

Fig. 2 Relationship between *Chromolaena odorata* cover and number of *Aegle marmelos* seedlings.

Shoot and root length

The shoot length of *A. marmelos* remained within the range of 5 to 6.5 cm across different treatments including leachate and water stress (Fig. 3A). Intermittent irrigation did not result in a statistically significant reduction in shoot length, whether treated with water (t = 0.831, $p = 0.418$) or leaf leachate (t = 0.956, $p = 0.353$) or root leachate (t = 0.574, $p = 0.574$) (Fig. 3A, Table S1). The toxicity of leaf leachate ($p = 0.999$ in drought, $p = 0.999$ in regular irrigation) and root leachate ($p = 0.931$ in drought, $p = 0.846$ in regular irrigation) did not show any effect on shoot length (Fig. 3A, Table S2).

Roots of seedlings irrigated regularly were longer compared to the seedlings that were treated with water stress irrespective of the use of water (t = 2.73, $p = 0.014$), leaf leachate (t = 3.005, $p = 0.008$), and root leachate (t = 2.915, $p = 0.010$). In the intermittent irrigation, root length was 12.25 ± 1.03 cm in root leachate compared to control (20.45 ± 0.87 cm) ($p < 0.001$) and leaf leachate (16.12 ± 0.48 cm) ($p = 0.003$) (Fig. 3B, Table S2). The root length decreased further by the root leachate compared to leaf leachate under the treatment of both intermittent irrigation ($p = 0.008$) and regular irrigation ($p = 0.036$) (Fig. 3B, Table S2). Similar to intermittent irrigation, when seedlings were regularly irrigated, roots were shorter with root leachate (15.98 ± 0.75 cm) compared to both control (23.47 ± 0.67 cm) ($p < 0.001$) and regular irrigation of leaf leachate (18.58 ± 0.655 cm) ($p < 0.001$) (Fig. 3B, Table S2).

Biomass and specific leaf area

Intermittent irrigation reduced the biomass of seedlings irrespective of the use of regular irrigation with water (t = 23.67, $p < 0.001$), leaf leachate (t = 8.39, $p < 0.001$), and root leachate (t = 28.56, $p < 0.001$) (Fig. 4A, Table S1). Average biomasses of seedlings during regular irrigation with water, leaf leachate and root leachate were 0.64 ± 0.004 g, 0.53 ± 0.02 g and 0.44 ± 0.01 g, respectively, whereas the biomass under intermittent irrigation was reduced approximately 50% or more than the biomass observed in the seedlings which were exposed to regular watering (Fig. 4A). The toxicity of leachates was distinct on biomass of *A. marmelos* under intermittent irrigation with significant effect observed for both leaf leachate ($p = 0.010$) and root leachate ($p < 0.001$) (Fig. 4A, Table S2).

SLA of the seedlings showed contrasting results. The seedlings subjected to intermittent irrigation with water had higher SLA compared to the seedlings irrigated regularly (t = –9.695; $p < 0.001$). However, the SLA of seedlings treated with leaf leachate (t = –1.325; $p = 0.217$) and root leachate (t = –0.732; $p = 0.474$) did not show any significant

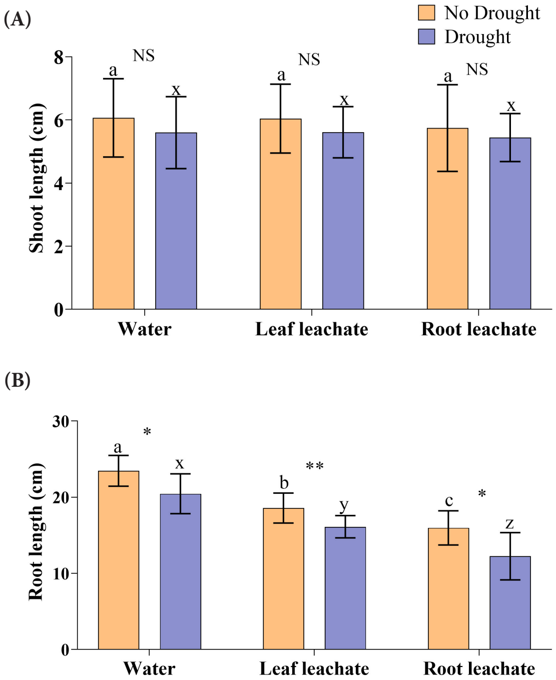

Fig. 3 Effect of drought and *Chromolaena odorata* leachate on (A) shoot length and (B) root length. The data are mean ± standard error. Letters above the error bars indicate significant differences among the treatments (no irrigation, intermittent irrigation, regular irrigation) obtained from one-way ANOVA ($p < 0.05$). The significant differences between sub-treatments (water and leachate irrigation) obtained from t-test are denoted by *$p < 0.05$, **$p < 0.01$. NS: not significant.

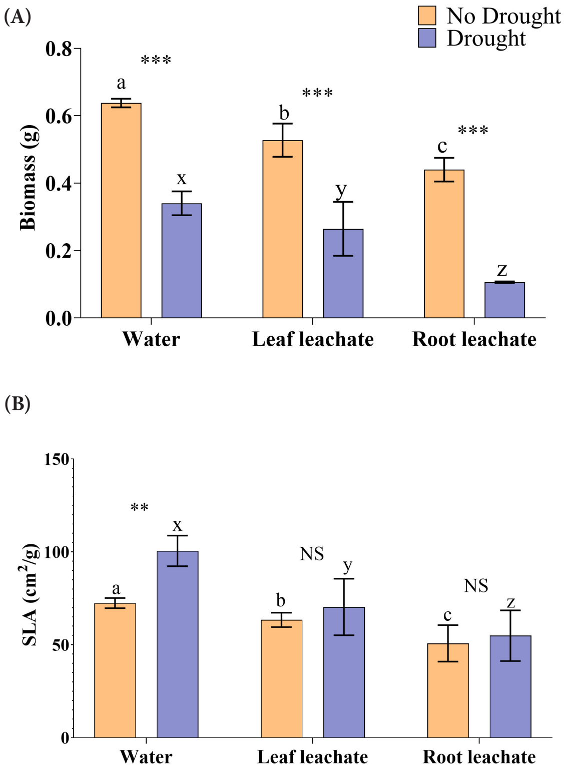

Fig. 4 Effect of drought and *Chromolaena odorata* leachate on (A) biomass and (B) specific leaf area (SLA). The data are mean ± standard error. Letters above the error bars indicate significant differences among the treatments (no irrigation, intermittent irrigation, regular irrigation) obtained from one-way ANOVA ($p < 0.05$). The significant differences between sub-treatments (water and leachate irrigation) obtained from t-test are denoted by **$p < 0.01$, ***$p < 0.001$. NS: not significant.

variation in terms of low moisture condition (Fig. 4B, Table S1). The SLA ranged from 50 to 75 cm^2/g in control and from 50 to 100 cm^2/g in intermittently irrigated pots (Fig. 4B).

The ANOVA showed that the SLA of the seedlings decreased significantly by *C. odorata* leaf leachate ($p = 0.014$) and root leachate ($p < 0.001$) during regular irrigation. Root leachate was more toxic than the leaf leachate as the SLA was significantly lower under root leachate than the leaf leachate ($p < 0.001$) (Fig. 4B, Table S2). In the case of intermittent irrigation, similar effects were observed ($p < 0.001$). Root leachate reduced the SLA much more than leaf leachate ($p = 0.042$) (Fig. 4B, Table S2).

Number of leaves and secondary roots

The number of leaves in seedlings did not vary when subjected to low moisture levels with water ($z = 0.156$, $p = 0.119$), leaf leachate ($z = 1.698$, $p = 0.089$) and root leachate ($z = 1.170$, $p = 0.087$) (Fig. 5A, Table S3). Neither there was significant variation in leaf number with leaf and root leachate of *C. odorata* ($p > 0.005$). The average number of leaves was between 2.89 ± 0.2 to 5.11 ± 0.35 (Fig. 5A, Table S4). A similar result was obtained while counting the number of secondary roots ($p > 0.005$, t-test and one-way ANOVA). The number of secondary roots ranged from 30 ± 1.08 to 36.89 ± 2.71 (Fig. 5B). Statistical details are given in Table S4.

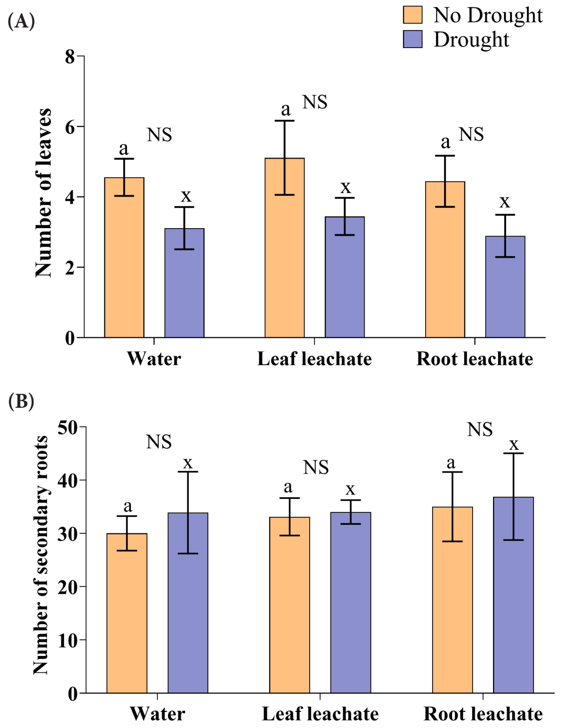

Fig. 5 Effect of drought and *Chromolaena odorata* leachate on (A) number of leaves and (B) number of secondary roots. The data are mean ± standard error. Letters above the error bars indicate significant differences among the treatments (no irrigation, intermittent irrigation, regular irrigation) obtained from Poisson regression ($p < 0.05$). The significant differences between sub-treatments (water and leachate irrigation) obtained from Poisson regression are denoted by NS. NS: not significant.

Proline and photosynthetic pigments

Photosynthetic pigments and proline content were measured in the leaves of harvested plants. Proline concentration was increased, when the seedlings were exposed to intermittent irrigation with water ($t = -12.708$, $p < 0.001$), leaf leachate ($t = -19.379$, $p < 0.001$), or root leachate ($t = -7.172$; $p < 0.001$) (Fig. 6A, Table S1). Seedlings exhibited significantly higher proline content under the stress of *C. odorata* leaf leachate ($p < 0.001$) and root leachate ($p < 0.001$) compared to the seedlings that were grown in control. Moisture deficiency with leaf leachate showed a highly pronounced increment of proline (Fig. 6A, Table S2).

The concentration of proline in regularly irrigated conditions with water was 0.789 ± 0.008 μmoles/g. Almost

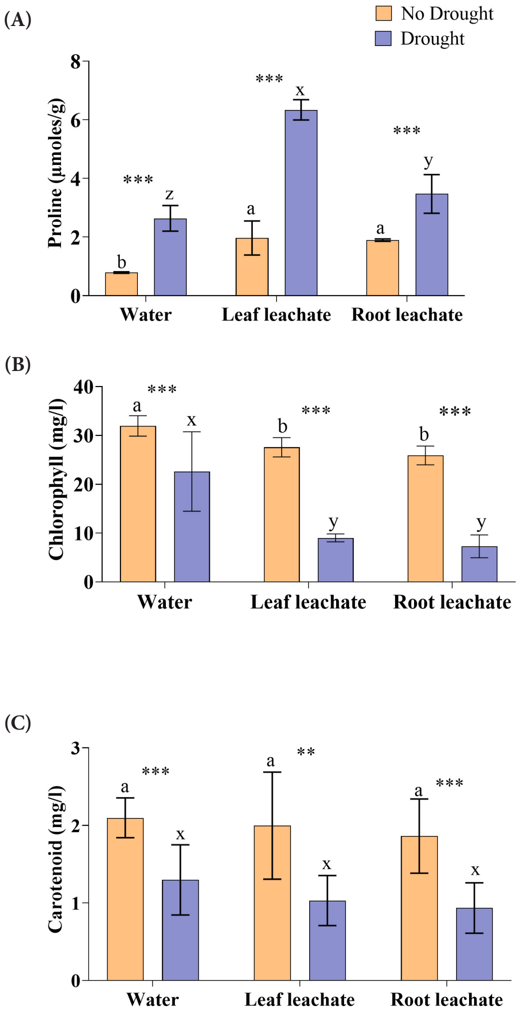

Fig. 6 Effect of drought and *Chromolaena odorata* leachate on proline (A), chlorophyll (B) and cartenoid (C). The data are mean ± standard error. Letters above the error bars indicate significant differences among the treatments (no irrigation, intermittent irrigation, regular irrigation) obtained from one-way ANOVA ($p < 0.05$). The significant differences between sub-treatments (water and leachate irrigation) obtained from t-test are denoted by $^{**}p < 0.01$, $^{***}p < 0.001$. NS: not significant.

similar concentration was found in the leaves treated with leaf leachate (1.97 ± 0.19 μmoles/g) and root leachate (1.89 ± 0.01 μmoles/g) when irrigated regularly (Fig. 6A). The concentrations increased by three folds under low moisture in soil with water and leaf leachate and by almost two folds in root leachate stress (Fig. 6A).

In case of photosynthetic pigments, chlorophyll content was 31.97 ± 0.69 mg/L, 27.58 ± 0.66 mg/L, and 25.91 ± 0.64 mg/L, under the treatment of water, leaf leachate and root leachate, respectively in the seedlings with regular irrigation (Fig. 6B). The chlorophyll content dropped while the seedlings grown under low moisture level in soil (22.62 ± 2.71 mg/L in intermittent irrigation by water; 9.01 ± 0.27 mg/L and 7.29 ± 0.78 mg/L in intermittent irrigation by leaf leachate and root leachate) (Fig. 6B).

Statistical analysis showed that there was a significant decrease in chlorophyll content by both root leachate and leaf leachate ($p < 0.001$) compared to water within regular and intermittent irrigations (Fig. 6B, Table S2). However, there was no variation in chlorophyll content in the seedlings subjected to the stress of root and leaf leachate under both regular irrigation ($p = 0.235$) and intermittent irrigation ($p = 0.739$) (Fig. 6B, Table S2). Intermittent irrigation was found to be inhibitory to the chlorophyll content with water ($t = 3.338$, $p = 0.009$), leaf leachate ($t = 25.976$, $p = 0.004$), and root leachates ($t = 18.41$, $p < 0.001$) (Fig. 6B, Table S2).

When plants were regularly given water, leaf leachate, and root leachate, the average carotenoid content was 2.09 ± 0.08 mg/L, 1.99 ± 0.23 mg/L, and 1.86 ± 0.16 mg/L, respectively (Fig. 6C). When they were subjected drought, the average carotenoid content was reduced to 1.29 ± 0.15 mg/L, 1.03 ± 0.11 mg/L and 0.93 ± 0.11 mg/L respectively (Fig. 6C). Statistically, unlike the chlorophyll content, carotenoid in the leaves of *A. marmelos* seedlings were not decreased by leachates of *C. odorata* ($p > 0.005$) (Fig. 6C, Table S2). However, drought treatment has significantly decreased the carotenoid content when irrigated with water ($t = 4.621$; $p < 0.001$), leaf leachate ($t = 3.806$; $p < 0.001$), and root leachate ($t = 4.800$; $p < 0.001$) (Fig. 6C, Table S1).

Discussion

The study revealed that a significant reduction in the number of seedlings of *A. marmelos* in its habitat due to increasing invasive *C. odorata* cover. Linear regression shows a significant negative relationship between the seedling density of *A. marmelos* and *C. odorata* cover. Within the range of *C. odorata* cover from 15% to 80% the seedlings showed a sharp decline in number with an increment of cover (Fig. 2). The result indicates the detrimental consequences of *C. odorata* invasion on natural seedling recruitment of such a valuable native tree.

The field assessment was similar to the study carried out by Thapa et al. (2016) who reported a declining trend of seedlings of native *Shorea robusta* with increasing *C. odorata* cover. Another study conducted by Gbètoho et al. (2018) in West Africa on the impact of this weed on native tree regeneration showed a strong and negative correlation with *C. odorata* cover (Gbètoho et al. 2018). Generally, earlier growth of the light-demanding species slows down and seedling mortality speeds up in the habitat with high cover of *C. odorata* (Honu and Dang 2000). The test species in our experiment, *A. marmelos* as a light-demanding species, the seedlings might be competing for light with *C. odorata* and there may be high seedling mortality in the invaded sites.

Besides competition for light, seedlings of *A. marmelos* have to adapt to high salinity and low soil moisture if they are growing in dry environments. And, if the dry habitats are invaded by *C. odorata,* the seedlings of *A. marmelos* also have to face the stress of the released products from aerial or belowground parts of *C. odorata* in the form of leachate. In this situation, the seedlings of *A. marmelos* have to respond to dual stresses, one is drought and another is an invasive plant's leachate. Evaluating the effects of these stresses on the seedlings of *A. marmelos* was the major concern of this study.

The native *A. marmelos* in the South-Asian region has been explained as the plants that prefer to grow in relatively dry conditions characterized by high temperature and low precipitation (Poudel and Chettri 2021; Sharma et al. 2013; Singh et al. 2014). On the other hand, many studies have shown that drought (salinity) can significantly decrease seed germination, seedling growth, and other qualitative and quantitative morphological characteristics (Singh and Chauhan 2020; Singh et al. 2015). Our study also showed the inhibitory effects of drought on *A. marmelos* seedling growth parameters (length and biomass, number of leaves and secondary roots, and photosynthetic pigments) (Figs. 3-6, Tables S1-S4). Roots, seedling biomass, and photosynthetic pigments exhibited high sensitivity to drought because these parameters decreased during drought conditions (Figs. 3B, 4A, 6B, 6C). These findings indicate the pronounced negative impact of water stress.

Interestingly, elevated drought levels led to an increase in the level of SLA and proline (Figs. 4B, 6A). Furthermore, the drought did not reduce shoot length, leaf number, and root numbers (Figs. 3A, 5A, 5B). It is conceivable that the plant may undergo specific adaptive responses under limited water conditions as a survival mechanism. Increased SLA accelerates plant growth rates, increases leaf biomass, and compensates loss of photosynthetic pigments in many species (Cornelissen et al. 2003; Gallagher et al. 2015). In addition, proline plays a crucial role in maintaining turgidity, preventing electrolyte leakage, and stabilizing membranes in cells which enable plants to withstand stress without experiencing oxidative bursts (Hayat et al. 2012). Hence, increased SLA and proline are the adaptive responses of *A. marmelos* that may contribute to the plant's ability to thrive in limited soil moisture conditions.

The major concern of this study, as mentioned above, was to depict the impacts of *C. odorata* to *A. marmelos* with simultaneous drought treatments. As expected, the inclusion of *C. odorata* leachate reduced *A. marmelos* root length, plant biomass, and photosynthetic pigments (Figs. 3B, 4A, 6B, 6C, Table S1). The presence of leachate significantly diminished both roots and seedling biomass, highlighting the toxicity of *C. odorata* on *A. marmelos* seedlings. This outcome suggests that *C. odorata* has distinct adverse impacts on *A. marmelos* by leaching the substances detrimental to the early stage of development.

The leachate treatment was the simulation of a natural mechanism wherein harmful allelochemicals from invasive plants are washed by rainwater, subsequently posing a threat to native plants. Kato-Noguchi and Kato (2023) identified more than 20 allelochemicals in *C. odorata* such as 1,8-cineole, which targets the root system (Kato-Noguchi and Kato 2023). Thapa et al. (2021) also identified various phytotoxic compounds like linalool, β-pinene, 1,3-cycloheptadiene, β-cubebene, cinnamaldehyde, and caryophyllene oxide in *C. odorata* leaf volatiles (Thapa et al. 2021). These compounds are known to hinder plant growth (Hu and Zhang 2013). Benzoic acid and cinnamic acid derivatives found in the leaves of *C. odorata*, have been associated with cellular damage and inhibition of overall growth as highlighted by Nandi and Mandal (2009).

Due to the evident toxicity of both drought and leachate, particularly affecting the roots of *A. marmelos* (Fig. 3B), their influence could potentially disrupt the root-shoot ratio, leading to a prominent long-term consequence. For example, a reduced root system may weaken plant stability and increase the risk of falling (Postma et al. 2014). As highlighted by Batish et al. (2006) toxicity of leachate on chlorophyll contents (Fig. 6B, C) hinders plants' photosynthetic efficacy (Batish et al. 2006). Comparatively, seedlings of *A. marmelos* exhibited lower SLA in normal conditions (no drought) but the addition of leachate contributed more pronounced reduction in the SLA (Fig. 4B). This decrease in SLA is associated with lower surface area which could have implications for plant physiology and growth (Evans and Poorter 2001).

The critical observation is that, under regular irrigation conditions, both root and leaf leachate exceeded the accumulation of proline in the seedlings compared to water irrigation (Fig. 6A). This suggests that endogenous proline accumulation might aid in recovering potential damage from both leachate and water stresses. The proline concentration was high in both regular irrigation and intermittent irrigation under *C. odorata* leachate (Fig. 6A). The increased concentration of proline serves as an indicative marker (Mansour and Ali 2017), suggesting that the plants are experiencing stressful conditions under the leachate as shown by the seedlings irrespective of its irrigation patterns (Fig. 6A). Nevertheless, some studies report that an excessive exogenous supply of proline may lead to toxic effects (Hayat et al. 2012; Heuer 2003; Jain et al. 2001).

All the imbalances outlined above carry significant implications for vital physiological processes such as root functioning, nutrient acquisition, and overall plant health (Hamblin and Tennant 1987; Zakaria et al. 2020). On one side, the increasing prevalence of drought poses a substantial challenge to the resilience of native plant seedlings, impacting their development and survival (Gómez-Aparicio et al. 2008; Kolb et al. 2020). Concurrently, invasive species are intensifying these threats. The declining population of *A. marmelos* has led to its classification as a 'near threatened species' on the IUCN Red List. Our study indicates that continued stresses could intensify the threats and the status of the plants may be 'threatened' soon.

The experimental evidence from our study indicates that the prolonged drought significantly impacts *A. marmelos*, with the observed effects on growth parameters being more severe than those observed under the drought. The addition of leachate further worsens the severity of the impact. In response to the stress, *A. marmelos* has strived to adapt by altering its SLA and increasing the proline content. However, these modifications alone may not be adequate to mitigate the stresses. While adding the leachate of *C. odorata,* the experiments showed intensification of the negative impacts on *A. marmelos* seedlings. Here, the critical concern should be how to protect the seedlings of such a valuable tree from invasion impacts. The leachates are commonly released from aerial parts like leaves by rainwater. In addition, the deposition of dry leaves and litter may also contribute to leaching the harmful components in the soil. Furthermore, roots of *C. odorata* continuously leach harmful substances, even if the aerial parts dry up during the summer. Based on our results, *C. odorata* from invaded habitats should be removed with root system for preventing its products from entering the soil where *A. marmelos* seedlings are growing.

It is noteworthy that *C. odorata* typically sprouts during March and forms a dense canopy until the rainy season ends in Nepal. Therefore, we recommend the removal of *C. odorata* before the sprouting season and before the onset of rainfall. Since *C. odorata* possesses underground tuber responsible for regenerating aerial parts, complete removal with underground parts is essential. In summary, the invasion of *C. odorata* can accelerate the decline of threatened species and it can be anticipated that it may lead to extinction after persistent and long-term invasions.

Hence, it is well known that allelochemicals from *C. odorata* are harmful to neighboring plants but not all the recipient plants may respond in the same way to the chemical stress. Different native species may respond differently depending on varied environmental factors. As soil moisture condition is one of the crucial factor for plant growth and development, its interaction with invasive plant products (leachates or allelochemicals in the leachates) is the matter of exploration. Information on such interaction would have significance in determining how invasive plants impacts native plant communities at particular environmental conditions.

Conclusions

This study highlights a significant decrease in the native *A. marmelos* population within its habitat under the invasion of *C. odorata.* The tree struggles with the combined stresses of both drought and *C. odorata* invasion in its dry habitat. This study concludes that a pronounced negative impact of water stress, however, the observed increase in SLA and proline content in the seedlings suggests potential adaptations enhancing the plant's resilience in conditions of limited soil moisture. Furthermore, the introduction of *C. odorata* leachate resulted in the reduction of *A. marmelos* root length, plant biomass, and chlorophyll content, indicating distinct adverse impacts on the early development of *A. marmelos* due to leached substances from *C. odorata*.

The evident toxicity of both drought and leachate on roots of *A. marmelos* can disrupt the root-shoot ratio, leading to a prominent long-term consequence. Additionally, the reduced chlorophyll content, pronounced reduction in the SLA, and elevated proline accumulation in the seedlings that are exposed to *C. odorata* leachate have implications for vital physiological processes and overall plant health. With the water stress, *C. odorata* intensifies the threats to the 'Near Threatened' *A. marmelos* which is a critical concern. In this regard, the better options to minimize the release of leachates from *C. odorata* are the removal of the weed from invaded habitats before its sprouting and before the onset of rainfall to prevent releasing the products from entering the soil where *A. marmelos* seedlings are growing. Based on these results, it is recommended that this weed be removed before monsoon to minimize its negative effects on valuable native plants like *A. marmelos*. This insight into the interaction between *C. odorata* invasion and drought effects on *A. marmelos* is crucial for developing focused conservation strategies to mitigate risks faced by this high-valued plant species.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.5141/jee.24.072.

Table S1. t-test statistics on comparison of growth between 'intermittent irrigation (drought)' and 'regular irrigation (control)'. **Table S2.** One-way ANOVA statistics among water, leaf leachate, and root leachate in 'intermittent irrigation (drought)' and 'regular irrigation (control)' treatments. **Table S3.** Poisson regression between 'intermittent irrigation (drought)' and 'regular irrigation (control)' treatments. **Table S4.** Poisson regression among water, leaf leachate, and root leachate. **Fig. S1.** (A) *Chromolaena odorata* invasion in the study site. (B) *Chromolaena odorata* and *Aegle marmelos* seedlings in the field. (C) *Aegle marmelos* seedlings growing in pots (pot experiment). **Fig. S2.** Harvested seedlings of *A. marmelos*. (A) Regular watering (normal water). (B) Regular irrigation (*Chromolaena odorata* leaf leachate). (C) Intermittent irrigation (normal water). (D) Intermittent irrigation (*C. odorata* leaf leachate). (E) Regular irrigation (*C. odorata* root leachate). (F) Intermittent irrigation (*C. odorata* root leachate).

Abbreviations

SLA: Specific leaf area
IUCN: International Union for Conservation of Nature
DW: Distilled water

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Authors' contributions

SP performed the experiments, analyzed data and wrote the first draft. LBT, SP, RRP, and MKC conceptualized research and experimental design. LBT, RRP, and MKC supervised the research, revised the manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable requests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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