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Research article

Preliminary bioactivity study of comfrey and azalea solutions on red spinach germination

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Abstract

Importance of the work: Allelopathy allows certain plants to influence the growth of neighboring species through chemical release. While azale (*Rhododendron simsii*) and comfrey (*Symphytum officinale*) are known for their allelopathic effects, their impact on vegetable crops like red spinach remains unclear. This warrants further investigation into their potential use in sustainable cultivation systems.

Objectives: To determine the bioactivity of comfrey and azalea on the growth of red spinach seeds.

Materials and Methods: A completely randomized design was used with two treatment factors: allelopathic plant type (comfrey and azalea) and solution concentration (25%, 50% and 100%). The control was distilled water.

Results: Comfrey and azalea had lethal concentration 50% values greater than 100%. Comfrey and azalea concentrations of 25−100% affected almost all measured germination growth parameters. Comfrey and azalea at 25-50% inhibited hypocotyl and cotyledon growth, but at 100% stimulated radicle, cotyledon and biomass growth. Azalea had stronger inhibition activity and weaker stimulatory activity than comfrey.

Main finding: Comfrey and azalea at 25−100% concentrations had no phytotoxicity activity. Comfrey and azalea at 25-50% had inhibitory activity, while at 100% they had stimulatory activity. Both test plants had inhibitory and biostimulant activities; this, they have potential for utilization and development as bioherbicides and plant growth regulators. Further research should investigate the inhibitory, stimulatory and allelochemical profiles of comfrey and azalea.

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Introduction

Red spinach (*Amaranthus tricolor* L.) is a vegetable plant that has a variety of benefits besides being a source of iron; it also has many desirable properties such as being antipyretic and analgesic, as well as being suitable for preventive therapy of obesity through regulating blood glucose levels and body mass index (Putri et al., 2016). Red spinach contains quercetin, rutin, isoquercetin, tannin and saponin compounds (Sarker et al., 2024). Red spinach is usually planted in an intercropping system with other vegetable crops or even with ornamental plants (Ribeiro et al., 2017). Increasing the production of red spinach can be done by improving cultivation techniques or methods. Various efforts have been made to improve red spinach cultivation methods, including proper fertilization, irrigation management, planting time, harvesting methods, and weed management (Ribeiro et al., 2018; Casini et al., 2022).

Red spinach can be planted together with other plants. Planting two or more plants in the same field can cause interactions between plants that can be positive, negative, or neutral, being expressed as intraspecific competition, interspecific competition or facilitation interactions (Kusumawati, 2018; Baucom et al., 2020; Fitri et al., 2021; Losapio, 2023). In addition, some plants contain allelochemicals as a plant response to environmental ecosystems that can affect the metabolic processes and growth of plants around them (Quan et al., 2022). Allelochemicals can cause damage to the plasma membrane of other plants, causing metabolic processes and cell function disruption, as well as regulating the growth of other plants positively, or negatively, and can cause damage to the surrounding soil (Scavo et al., 2019). Allelochemicals can be useful for controlling weeds around plants, acting as natural pesticides (Quan et al., 2022). Species in the genus *Rhododendron* have various compounds that have the potential for allelopathy, with one of them being catechin (Wang et al., 2013). Allelopathy in these plants is released and affects plants and microorganisms through soil and litter exudation. For example, allelopathy in *Rhododendron* spp. accumulates in senesced plant leaves and acts via decomposition of plant litter (Wang et al., 2022).

Comfrey (*Symphytum officinale* L.) exhibits allelopathic effects through the release of allelochemical compounds that influence surrounding plants, and potentially affect humans and animals. Its extracts have been shown to inhibit fungal growth and activate natural plant defense mechanisms (Nasir et al., 2005). Several studies have shown that this plant has the ability to biocontrol against pests and diseases (Rocha et al., 2009). *Rhododendron simsii* Planch. is an azalea used as an ornamental plant that releases allelochemical with moderate allelopathic effects that depend on the mode of exposure to the ecosystem. For example, leachates from azalea tissues have slightly negative effects, whereas direct exposure to azalea residues causes a stronger negative impact (Sutton and Wilkinson, 2007; Esen et al., 2006). Azalea can be used as a soil improver with a milder effect (Quan et al., 2022). Research has shown that ethanol extract applications of comfrey inhibited radicle growth in lettuce by up to 66% (Nasir et al., 2005). While no published studies are known regarding the allelopathic effect of *R. simsii*, research on another species of this genus (*Rhododendron arboreum*) showed that under *in vitro* conditions, its leaf extract significantly inhibited the germination and development of *Eleusine coracana* and *Glycine max* sprouts through reduction of radicle and plumula lengths, while its bark extract inhibited and stimulated germination and growth and in pot culture, both extracts produced varied responses to both plants (Rana et al., 2024).

The aim of the current study was to investigate the growth response of red spinach to allelopathy produced by *R. simsii* and *S. officinale* extracts and to determine the most suitable concentration of both.

Materials and Methods

This research was conducted at the Traditional Medicine Raw Materials Laboratory, Research Center for Medicinal Raw Materials and Traditional Medicines, National Research and Innovation Agency in Tawangmangu, Karanganyar Regency, Central Java, Indonesia from March to July 2024. A completely randomized design (CRD) was used with two treatment factors: 1) allelopathic plant species [comfrey (*S. officinale* L.) and azalea (*R. simsii*)]; and 2) extract concentrations (25%, 50% and 100%). The control treatment consisted of just distilled water. Each treatment used 20 red spinach seeds with three replications. The research materials consisted of comfrey (fresh leaves), azalea (fresh leaves and flowers) and "Panah Merah, East West Seed" red spinach seeds with 85% germination, 99% purity and 8% maximum moisture content. The equipment used included Petri dishes, a magnifying glass, tweezers, a manual pipette, weight scales and a ruler.

The solution was prepared by pulverizing the separate comfrey and azalea samples by squeezing. Then, 125 g of the resulting material of each sample was immersed in 500 mL of distilled water (25% concentration), 250 g of the material in 500 mL of distilled water (50% concentration) and 500 g of the material in 500 mL of distilled water (100%). The material was immersed for 3 hr and then filtered to obtain the different comfrey and azalea solutions.

For each sample, a Petri dish was covered with two layers of filter paper and 20 red spinach seeds were arranged evenly on the filter paper and treated with 8 mL of solution, according to the relevant treatment concentration (25%, 50%,100% or distilled water). The Petri dishes were placed in a laboratory room at 22.5±5°C with the relative humidity around 75%. As much as 2 mL of the respective solution was added every 48 hr to maintain filter paper humidity. The germinated numbers were counted from the treatment day until day 7 after treatment. Data were recorded on radicle length (RL), hypocotyl length (HL), cotyledon length (CL) and biomass weight per Petri dish (BM). The experiment method followed Li et al. (2021), with modifications.

The GerminaR and agricolae packages (Mendiburu, 2023; Lozano-Isla et al., 2019)in the R software (R Core Team, 2024) and the RStudio (Posit team, 2024) integrated development environment software for R were used to analyze the germination growth data: germination percentage (GRP), mean germination time (MGT), mean germination rate (MGR), germination speed coefficient (GSP), germination uncertainty index (UNC), germination synchrony (SYN), germination variance (VGT), standard deviation of germination time (SDG) and germination velocity coefficient (CVG).

The time taken to 50% germination (T50) was calculated based on Equation 1 (Catiempo et al., 2021):

$$\mathrm{T50} = \mathrm{ti} + \frac{\frac{\mathrm{N}}{2} - \mathrm{ni}\,(\mathrm{tj} - \mathrm{ti})}{(\mathrm{nj} - \mathrm{ni})} \qquad (1)$$

where N is the total number of germinated seeds; and nj and ni are the total numbers of seeds germinated by adjacent counts at times tj (days) and ti (days), respectively.

The allelopathy response index (RI) was calculated to quantify the type and intensity of allelopathy using the method described above and applying the criteria on Equation 2:

$$\text{if } T \geq C, \text{ then } RI = 1 - (C / T); \text{ and if } T < C, \text{ then } RI = (T / C) - 1 \qquad (2)$$

where C is the control value; T is the treatment value; RI > 0 is a stimulatory effect; and RI < 0 is an inhibitory effect.

Synthetic allelopathy (SE) was evaluated by calculating the arithmetic mean value of RI for seven parameters (germination rate, germination speed index, germination index, radicle length, hypocotyl length, cotyledon length and biomass), according to Scavo et al. (2022).

The percentage reduction in the shoot and root lengths of the red spinach seedlings caused by the allelopathic effect of the aqueous weed extracts was calculated based on Equation 3:

$$\text{Percentage reduction} = ((C - T) / C) \times 100 \qquad (3)$$

where C is the value obtained from the control treatment; and T is the value obtained from the extract treatment.

The average percentage inhibition (API) was used to analyze the overall impacts of plant extracts on the growth, based on Equation 4 (Lalbiak et al., 2022):

$$\mathrm{API} = \frac{\begin{array}{c}(\text{\% Radicle reduction}+\text{\% Hypocotyl reduction}\\ +\text{\% Cotyledon reduction}+\text{\% Biomass reduction}\\ +\text{\% T50 reduction})\end{array}}{5} \qquad (4)$$

where 5 is the total number of parameters covered.

The germination data, germination growth data, RI, SE, growth reductions and API data were subjected to analysis of variance for the factorial experiment in the CRD based on Duncan's multiple range test (DMRT) at $p \leq 0.05$ to identify any significant effects of treatment on germination, growth and allelopathic effects of plant solution and the concentration treatment factor on red spinach. Data on the number of live sprouts were subjected to probit analysis using the Excel software (Microsoft® Excel® for Microsoft 365 MSO, Version 2505) to determine the toxicity of treatments on red spinach. The T50 germination results were subjected to analysis of variance for the factorial experiment in the CRD based on DMRT at $p \leq 0.05$ to identify any significant differences in the time required for 50% seed germination. In addition, the germination growth data were analyzed for correlations between treatment factors and red spinach growth.

Results and Discussion

The different plant solution treatment factors had significant effects on germination, radicle length and hypocotyl length, but no significant effects on cotyledon length and biomass of red spinach (Table 1). The comfrey and azalea solutions decreased germination compared to the control. Mamlic et al. (2024) also reported that comfrey solution decreased germination energy and vigor index, which also affect the germination percentage. Germination in the comfrey treatment was not significantly different from the azalea treatment. The radicle length of red spinach in both solution types was lower than the control; however, the lowest radicle length was obtained in the comfrey solution. Both types of solution increased the hypocotyl length of red spinach compared to the control. The concentration difference treatment factor had highly significant effect on radicle length, decreasing the radicle length at concentrations higher than 25%. Furthermore, the concentration had a significant effect on hypocotyl length and cotyledon length. The highest hypocotyl length was obtained at 25% concentration and decreased as the concentration increased; however, all three tested concentrations were higher than the control. The highest cotyledon length was obtained using the 25% concentration, followed by the 50% concentration. The cotyledon length at 100% concentration was the same as the control. The difference in concentration did not significantly affect the germination and biomass of sprouts.

The plant solution treatment factor had different effects on the germination of red spinach seeds (Fig. 1A). The application of the comfrey solution treatment decreased the number of germinated seeds by about 20% compared to the control treatment on day 3 after treatment. In addition, until the end of observation, there was only a slight increase in the number of sprouts. In contrast, germination that occurred following the application of the azalea solution resulted in about a 40% decrease in the number of red spinach sprouts on day 3, then a 10% increase in the number of sprouts on days 4 and 5, followed by an increase by about 5% on days 6 and 7.

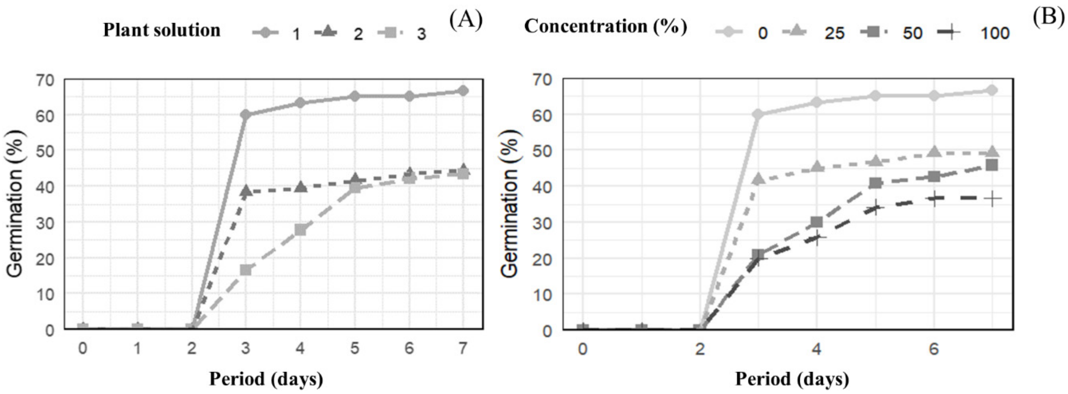

Fig. 1 Germination percentage over time of *Amaranthus tricolor* L. as response to allelopathic plant extract: (A) effects of plant species (comfrey and azalea); (B) effects of plant extract concentrations (25%, 50%, and 100%), with distilled water as the control (1).

Table 1 Effect on red spinach germination parameters of plant solution type and concentration treatments

	Germination	Radicle length (cm)	Hypocotyl length (cm)	Cotyledon length (cm)	Biomass/ Petri dish (g)
Plant solution					
Control	13.33±0.58 [a]	2.91±0.64 [a]	1.03±0.29 [b]	0.29±0.02 [a]	0.09±0.02 [a]
Comfrey	8.67±2.00 [b]	1.71±0.45 [c]	1.82±0.39 [a]	0.32±0.05 [a]	0.07±0.03 [a]
Azalea	8.56±2.79 [b]	2.19±0.75 [b]	1.59±0.39 [a]	0.29±0.08 [a]	0.08±0.03 [a]
Concentration					
Control	13.33±0.58 [a]	2.91±0.64 [a]	1.03±0.29 [c]	0.29±0.02 [b]	0.09±0.02 [a]
25%	9.83±1.94 [a]	2.67±0.58 [a]	2.06±0.31 [a]	0.36±0.05 [a]	0.09±0.02 [a]
50%	8.67±2.42 [a]	1.79±0.17 [b]	1.68±0.29 [ab]	0.30±0.04 [ab]	0.07±0.03 [a]
100%	7.33±2.34 [a]	1.39±0.24 [b]	1.37±0.27 [bc]	0.27±0.08 [b]	0.06±0.03 [a]

Values (mean ± SD) in a column for each factor followed by different lowercase superscripts are significantly ($p < 0.05$) different, based on Duncan's multiple range test.

Fig. 1B shows that increasing the concentration of the solution decreased the number of red spinach sprouts. The type of solution material (comfrey or azalea) and the different concentrations used (25− 100%) did not cause 50% mortality of treated red spinach seeds. The probit log (in parts per million, ppm) results showed that the value for the lethal concentration of 50% (LC50) for the comfrey solution on the red spinach seeds was 6.27 log (ppm) or 185.67% compared to the LC50 values of the azalea solution on the red spinach seeds was 6.39 log (ppm) or 245.72% (Fig. 2). It was assumed that both plant solutions could inhibit red spinach germination due to the toxicity effects of their allelochemicals (Weya et al., 2013). Comfrey contains allelochemical compounds in the form of pyrrolizidine alkaloids and rosmarinic acid (Kimel et al., 2023; Fujii, 2016). Generally, plants that contain pyrrolizidine alkaloids become invasive weeds (Fujii, 2016). Comfrey has potential as a candidate allelopathic ground cover crop (Nasir et al., 2005; Fujii, 2016).

Azalea contains quercetin 3-O-a-L-rhamnoside, tamarixetin 3-rhamnoside, isoquercitrin, hyperoside, myricetin-3-O-a-L-rhamnopyranoside, afzelin, quercetin-3-O-a-L-arabinofuranoside, quercetin-3-O-a-D-arabinopyranoside, (+)-catechin, kaempferol, dihydromyricetin, quercetin, vanillic acid, gallic acid and farrerol (Wang et al., 2023). Compared to the comfrey solution and the control, the azalea solution resulted in a longer period for the red spinach to achieve 50% seed germination (Fig. 3). The 50−100% concentration differences in the treatments resulted in longer germination periods for the red spinach to achieve 50% seed germination than for the 25% concentration and the control. The DMRT tested of the effect of the combination of plant solution treatment and concentration difference, with the results indicating that the 100% and 50% azalea treatments required the longest period to achieve 50% germination of the tested red spinach seeds (4−5 d and 3−4 d, respectively), as shown in Fig. 4. Several *Rhododendron* species have been reported to inhibit germination indirectly because the leaf litter from these plants inhibited ectomycorrhizal activity, which could help to supply nutrition for its mutual plants (Clinton and Vose, 1996; Nilsen et al., 1999).

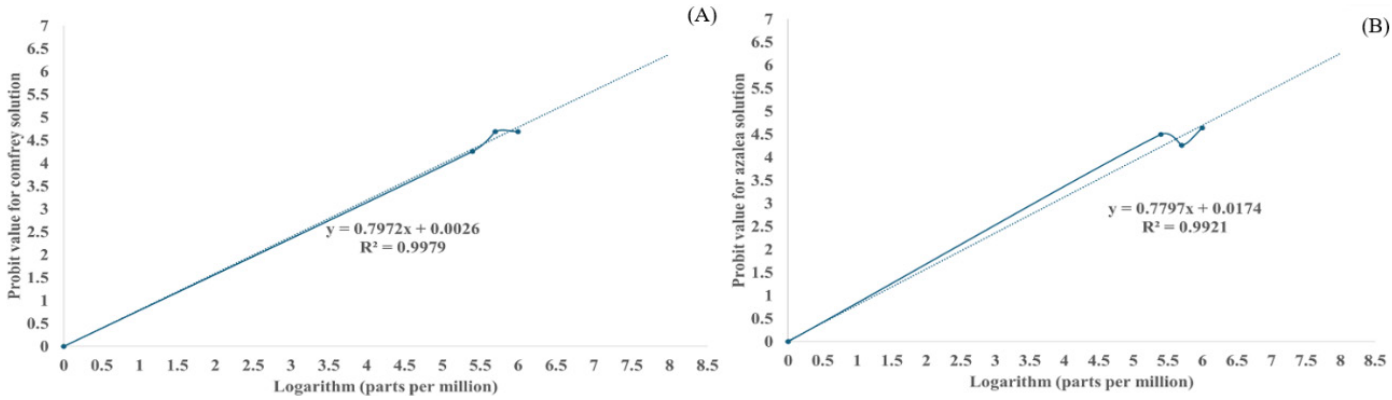

Fig. 2 Probit plots for Lethal consentration 50 (LC50) estimation: (A) effect of comfrey solutions on *Amaranthus tricolor* seeds; (B) effect of azalea solution on *A. tricolor* seeds. R^2 (the coefficient of determination), is a measurement of how well the statistical model predicts an outcome.

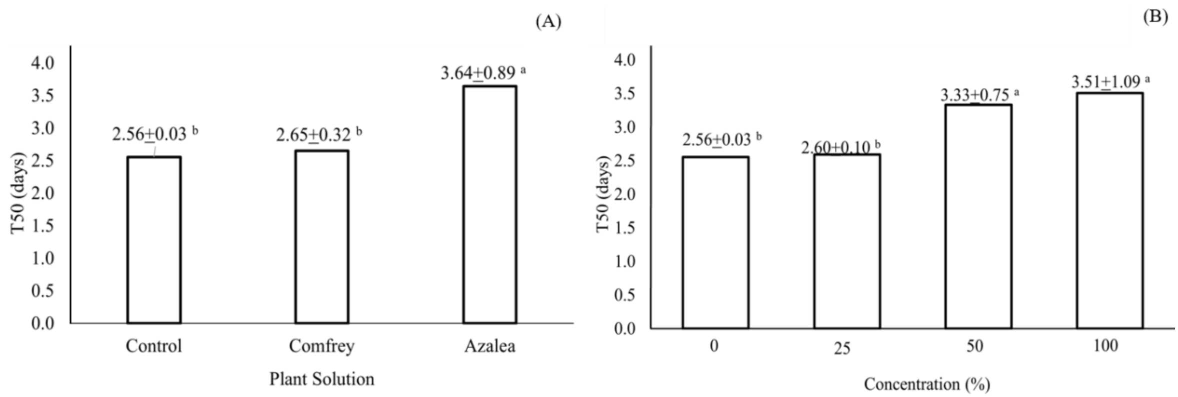

Fig. 3 Median germination time (T50) of *Amaranthus tricolor* based on: (A) different allelopathic plant extract treatments; (B): varying extract concentrations. Values (mean ± SD) within the same factor with different lowercase superscripts are significantly ($p < 0.05$) different, based on Duncan's multiple range test.

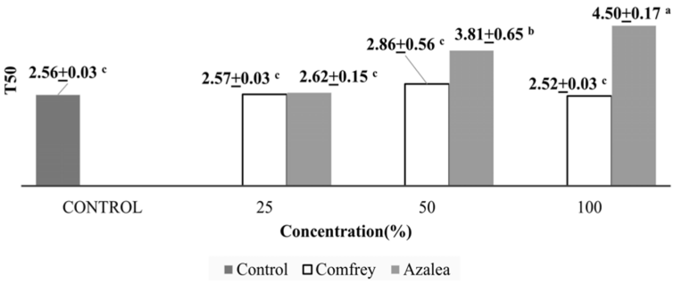

Fig. 4 Median germination time (T50) of *Amaranthus tricolor* under combinations of allelopathic plant species and extract concentration treatments. Bars represent mean and error bars represent ±SD. Different lowercase letters above bars indicate significant (p < 0.05) differences between mean values, based on Duncan's multiple range test.

The combination of the plant solution and concentration treatment factors only significantly affected the cotyledon length (Table 2) . The highest cotyledon length was produced by the 25% comfrey treatment, with the lowest from the 100% azalea treatment. The 25% azalea, 50% azalea and 100% comfrey treatments had cotyledon lengths that were not significantly different.

The plant solution treatment factor had a highly significant effect on some of the red spinach sprout growth parameters (GRP, MGT, MGR and GSP) but not on others (UNC, SYN, VGT, SDG and CVG), as shown in Table 3. The red spinach sprouts in the plant solution treatments had decreases in GRP compared to the control. The highest MGT value of red spinach was produced by the azalea treatment. The lowest MGR and GSP values were produced by the azalea treatment. The concentration treatment factor had a highly significant effect on MGT, MGR and GRP of the red spinach, as well as a significant effect on VGT of red spinach, but did not have a significant effect on GRP, UNC, SYN, SDG and on CVG. MGT values at 50% and 100% concentrations were higher than 25% concentration and control. While the MGR and GSP values of red spinach at 50% and 100% concentrations were lower than the 25% concentration and control. The highest VGT value was obtained at 50% concentration and the lowest at 100% concentration.

Table 2 Effect on red spinach cotyledon length (centimeters) of plant solution and concentration combination treatments

Plant solution	Concentration (%)			
	0	25%	50%	100%
Control	0.29±0.02 abc	-	-	-
Comfrey	-	0.37±0.06 a	0.28±0.02 bc	0.32±0.02 ab
Azalea	-	0.34±0.05 ab	0.32±0.05 ab	0.21±0.07 c

Values (mean ± SD) in a column within each factor followed by different lowercase superscripts are significantly (p < 0.05) different, based on Duncan's multiple range test.

Table 3 Effects on red spinach germination growth parameters of different plant solution types and concentration treatments

Treatment	GRP	MGT	MGR	GSP	UNC	SYN
Plant solution						
Control	66.67±2.89 a	3.20±0.16 b	0.32±0.02 a	31.29±1.55 a	0.38±0.39 a	0.80±0.08 a
Comfrey	44.44±10.14 b	3.34±0.43 b	0.30±0.03 a	30.30±3.49 a	0.00±0.47 a	0.75±0.27 a
Azalea	43.33±13.69 b	4.22±0.87 a	0.25±0.06 b	24.69±5.46 b	0.59±1.22 a	0.55±0.28 a
Concentration (%)						
Control	66.67± 2.89 a	3.20±0.16 b	0.31±0.02 a	31.29±1.55 a	0.51±0.23 a	0.80±0.08 a
25	49.17±9.70 a	3.27±0.22 b	0.31±0.02 a	30.69±1.97 a	0.58±0.40 a	0.74±0.18 a
50	45.83±11.58 a	4.12±0.75 a	0.25±0.05 b	25.00±4.86 b	1.27±0.66 a	0.45±0.29 a
100	36.67±11.69 a	3.96±1.05 a	0.27±0.06 b	26.78±6.90 b	0.48±0.63 a	0.78±0.28 a

Treatment	VGT	SDG	CVG
Plant solution			
Control	0.54±0.63 a	0.64±0.43 a	19.71±12.28 a
Comfrey	0.80±1.03 a	0.66±0.64 a	17.98±16.38 a
Azalea	0.55±0.46 a	0.62±0.42 a	14.71±9.91 a
Concentration (%)			
Control	0.54±0.63 ab	0.64±0.43 a	19.71±12.28 a
25	0.54±0.50 ab	0.63±0.41 a	18.74±11.64 a
50	1.27±1.00 a	0.99±0.58 a	23.34±14.55 a
100	0.21±0.37 b	0.3±0.39 a	6.95±8.80 a

GRP = germination percentage; MGT = mean germination time; MGR = mean germination rate; GSP = germination speed coefficient; UNC = germination uncertainty index; SYN = germination syncrony; VGT = germination variane; CVG = germination velocity coefficient . Values (mean ± SD) in a column within each factor followed by different lowercase superscripts are significantly (p < 0.05) different, based on Duncan's multiple range test.

Table 4 shows that the combination of plant solution and concentration treatments had a very significant effect on the MGT, MGR and GSP parameters of red spinach sprout growth, but did not affect other red spinach sprout growth parameters. The highest MGT values were obtained in the 50% and 100% azalea treatments. MGR and GSP values in the 25− 100% comfrey and 25% azalea treatments had values that were not significantly different from the control. The 50% and 100% azalea treatments had lower MGR and GSP values than the control.

Table 5 shows that the plant solution treatment had a highly significant effect on RI.MGT, RI.MGR, RI.GSP, RI.RL and RI.HL, but not on other variables. The values of RI.MGT and RI.RL in the azalea treatment were higher than for the comfrey treatment, while the values of RI.MGT, RI.GSP and RI.HL, in the comfrey treatment were higher than for the azalea treatment. The different concentration factors used had a highly significant effect on the variables RI.MGT, RI.MGR, RI.GSP, RI.RL, RI.HL, RI.GSP and RI.SYN, but not on the other variables. The RI.MGT values at the 50% and 100% concentrations were higher than those at the 25% concentration. The highest RI.MGR, RI.GSP, RI.RL, RI.HL, RI.GSP and SE values were at the 25% concentration. Based on the RI values, the plant solution treatment had an inhibitory effect on the variables GRP, MGR, GSP, UNC, SYN, VGT, CVG, RL, BM and SE and had a bio-stimulatory effect on the variables MGT and HL. The different concentrations used had an inhibitory effect on GRP, MGR, GSP, UNC, SYN, CVG, RL, CL, BM and SE, as well as a bio-stimulatory effect on MGT, VGT and HL.

Table 4 Effects on red spinach mean germination time (MGT), mean germination rate (MGR) and germination speed coefficient (GSP) of different plant solution types and concentration combination treatments

Variable	Plant solution	Concentration (%)			
		0	25	50	100
MGT	Control	3.20±0.16 [b]	-	-	-
	Comfrey	-	3.31±0.17 [b]	3.69±0.61 [b]	3.03±0.06 [b]
	Azalea	-	3.23±0.28 [b]	4.55±0.68 [a]	4.88±0.44 [a]
MGR	Control	0.31±0.02 [a]	-	-	-
	Comfrey	-	0.30±0.02 [a]	0.28±0.05 [a]	0.33±0.01 [a]
	Azalea	-	0.31±0.03 [a]	0.22±0.04 [b]	0.21±0.02 [b]
GSP	Control	31.29±1.55 [a]	-	-	-
	Comfrey	-	30.26±1.5 [a]	27.66±4.98 [a]	32.97±0.62 [a]
	Azalea	-	31.12±2.63 [a]	22.35±3.63 [b]	20.59±1.91 [b]

Values (mean ± SD) in a column within each factor followed by different lowercase superscripts are significantly ($p < 0.05$) different, based on Duncan's multiple range test.

Table 5 Allelopathy response index and synthetic allelopathy of red spinach growth parameters for different plant solution types and concentration treatments

Treatment	RI.GRP	RI.MGT	RI.MGR	RI.GSP	RI.UNC	RI.SYN	RI. VGT
Plant solution							
Comfrey	-0.33±0.15 [a]	0.03±0.11 [b]	-0.03±0.11 [a]	-0.03±0.11 [a]	-0.18±0.68 [a]	-0.08±0.32 [a]	-0.23±0.78 [a]
Azalea	-0.35±0.21 [a]	0.21±0.17 [a]	-0.21±0.17 [b]	-0.21±0.17 [b]	0.19±0.7 [a]	-0.32±0.34 [a]	-0.16±0.65 [a]
Concentration (%)							
25	-0.26±0.15 [a]	0.02±0.06 [b]	-0.02±0.06 [a]	-0.02±0.06 [a]	-0.03±0.53 [a]	-0.09±0.22 [a]	0.54±0.50 [a]
50	-0.31±0.17 [a]	0.20±0.15 [a]	-0.20±0.16 [b]	-0.20±0.15 [b]	0.38±0.68 [a]	-0.45±0.35 [a]	1.27±1.00 [a]
100	-0.45±0.16 [a]	0.14±0.22 [a]	-0.14±0.22 [b]	-0.12±0.22 [b]	-0.34±0.76 [a]	-0.06±0.33 [a]	0.21±0.37 [a]
	RI CVG	RI.RL	RI.HL	RI.CL	RI.BM	SE	
Plant solution							
Comfrey	-0.23±0.66 [a]	-0.41±0.15 [b]	0.41±0.12 [a]	0.09±0.13 [a]	-0.22±0.28 [a]	-0.44±0.61 [a]	
Azalea	-0.27±0.49 [a]	-0.25±0.25 [a]	0.31±0.17 [b]	-0.01±0.25 [a]	-0.14±0.34 [a]	-0.23±0.93 [a]	
Concentration (%)							
25	-0.11±0.51 [a]	-0.09±0.19 [a]	0.49±0.07 [a]	0.18±0.11 [a]	0.01±0.21 [a]	0.34±0.46 [a]	
50	0.02±0.55 [a]	-0.39±0.06 [b]	0.37±0.10 [b]	0.03±0.13 [ab]	-0.25±0.31 [a]	-0.36±0.59 [ab]	
100	-0.65±0.44 [a]	-0.52±0.08 [c]	0.22±0.13 [c]	-0.08±0.26 [b]	-0.31±0.32 [a]	-0.99±0.61 [b]	

RI = response index; SE = synthetic allelopathy; GRP = germination percentage; MGT = mean germination time; MGR = mean germination rate; GSP = germination speed coefficient; UNC = germination uncertainty index; SYN = germination syncrony; VGT = germination variane; CVG = germination velocity coefficient; RL = radicle length, HL = hypocotyl length; CL = cotyledon length; BM = biomass weight per petri dish. Darker blue color indicates allelopathic response index closer to 1 (stimulant effect), while darker red indicates allelopathic response index closer to -1 (inhibitory effect). Values (mean ± SD) in a column within each factor followed by different lowercase superscripts are significantly ($p < 0.05$) different, based on Duncan's multiple range test.

Based on the results from the analysis of variance, the combination of plant solution and concentration treatment had a significant effect on the RI.MGT, RI.MGR, RI.GSP, RI.RL and RI.CL red spinach sprouts growth variables; however, the results of further tests using DMRT showed that the treatment combination used did not have a significant effect on the five variables. Based on the magnitude of the RI value, both comfrey treatments and azalea treatments with concentrations of 25−100% only had a bio-stimulatory effect on MGT. In addition, the 25−100% comfrey treatments had a bio-stimulatory effect on CL, but not on the other variables (Table 6).

Fig. 5 shows that the allelopathic materials used to immerse red spinach seed were positively correlated with concentration. Specifically, the allelopathic materials were positively correlated with HL and negatively correlated to CL, RL and biomass, as well as being strongly negatively correlated with germination. Concentration has a strong-to-weak negative correlation with the growth parameters of RL, germination, biomass, CL and HL. The biomass of the sprouts has strong correlation with germination, CL and HL.

Fig. 6 shows that comfrey and azalea at a concentration of 25% caused moderate inhibition, with the inhibition decreasing as the concentration increased. The inhibition due to the 25% and 50% azalea concentrations was higher than for the 25% and 50% comfrey concentrations. The comfrey and azalea 100% concentrations both had a stimulating effect on red spinach germination, with 100% comfrey having a higher stimulant effect than 100% azalea. The API assessment of comfrey and azalea showed that concentrations in the range 25−50% inhibited red spinach germination growth, but at 100%

Table 6 Red spinach RI.MGT, RI.MGR, RI.GSP, RI.RL and RI.CL effected by plant solution and concentration combination treatments

Variable	Plant solution	Concentration (%)		
		25	50	100
RI.MGT	Comfrey	0.13±0.21 [a]	0.11±0.20 [a]	0.03±0.11 [a]
	Azalea	0.18±0.22 [a]	0.22±0.15 [a]	0.06±0.18 [a]
RI.MGR	Comfrey	0.13±0.22 [a]	-0.11±0.20 [a]	-0.03±0.11 [a]
	Azalea	-0.17±0.22 [a]	-0.22±0.15 [a]	-0.06±0.18 [a]
RI.GSP	Comfrey	-0.13±0.21 [a]	-0.11±0.20 [a]	-0.03±0.11 [a]
	Azalea	-0.18±0.22 [a]	-0.22±0.15 [a]	-0.07±0.1 [a]
RI.RL	Comfrey	-0.34±0.16 [a]	-0.41±0.22 [a]	-0.43±0.12 [a]
	Azalea	-0.26±0.33 [a]	-0.31±0.36 [a]	-0.25±0.22 [a]
RI.CL	Comfrey	0.06±0.12 [a]	0.20±0.12 [a]	0.13±0.09 [a]
	Azalea	-0.00±0.07 [a]	-0.11±0.41 [a]	-0.05±0.17 [a]

RI = response index; SE = synthetic allelopathy; MGT = mean germination time; MGR = mean germination rate; GSP = germination speed coefficient; RL = radicle length; CL = cotyledon length). Darker blue color indicates allelopathic response index closer to 1 (stimulant effect), while darker red indicates allelopathic response index closer to -1 (inhibitory effect). Values (mean ± SD) in a column within each factor followed by different lowercase superscripts are significantly ($p < 0.05$) different, based on Duncan's multiple range test.

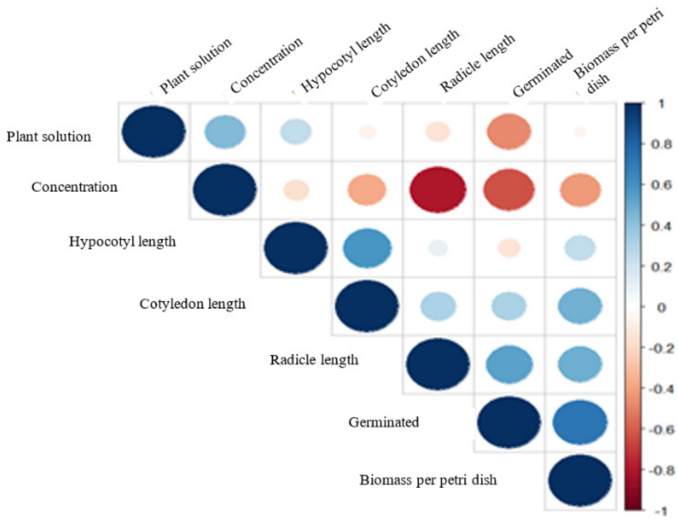

Fig. 5 Correlation graph of solution and concentration of comfrey and azalea on *Amaranthus tricolor* germination, where positive correlations displayed in blue and negative correlations displayed in red, with darker shading and larger-sized shapes indicating greater correlation.

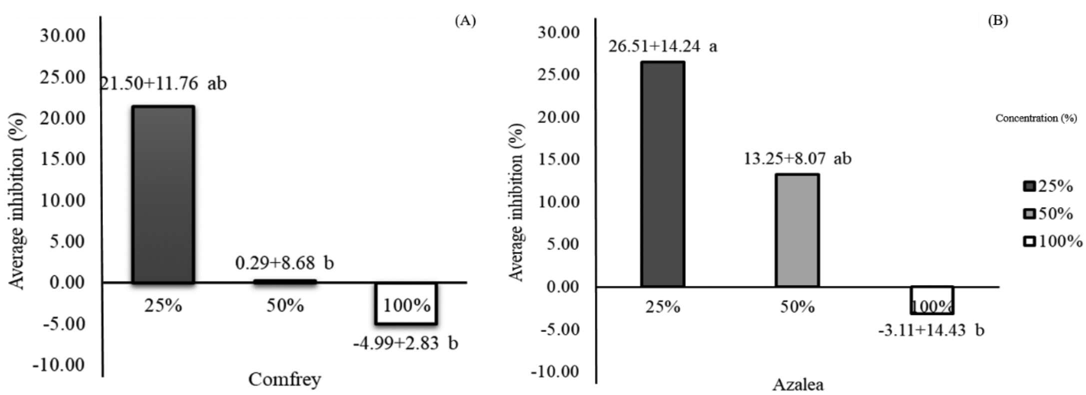

Fig. 6 Average percentage inhibition of *Amaranthus tricolor* germination and growth by aqueous extract of comfrey (left panel) and azalea (right panel). Bars represent mean values, error bars represent ±SD. Different lowercase letters above bars denote significant differences ($p < 0.05$) among mean values, based on Duncan's multiple range test .

stimulated some parameters regarding red spinach germination growth. No published references or research regarding the API of these two plants could be found.

Mihail et al. (2021) revealed that comfrey could be utilized as a renewable source of bio-stimulation to stimulate germination, growth and development (growth hormones). Comfrey contains rosmarinic acid and triterpenoids, while azalea contains the growth hormones polyamines, gibberellin and cytokinin (Tantivatana, 1986; Meijón et al., 2011). Secondary metabolites at low concentrations can act as inhibitors, but at high concentrations, they could become an integral part of the signaling network complex that regulates and modulates pathways to produce plant growth hormones (Erb and Kliebenstein, 2020) . Growth hormones are formed due to the biosynthesis of secondary metabolites as an adaptation to environmental stress (Bajguz and Piotrowska-Niczyporuk, 2023). Secondary metabolites, such as flavonoids, alkaloids and terpenoids, are synthesized through specific pathways that often overlap with primary metabolites and these compounds can affect the synthesis and activity of growth hormones such as auxin, gibberellin and cytokinin (Mahnert et al., 2018; Ningsih et al., 2023; Nabillah and Chatri, 2024). The interaction between secondary metabolites and growth hormones is bidirectional as growth hormones can induce the production of secondary metabolites, which in turn can regulate hormone levels and activity; this feedback loop ensures the plant can adapt (Diver, 1999; Savić et al., 2015; Susanti et al., 2022; Ningsih et al., 2023; Nabillah and Chatri, 2024). The currents result showed that comfrey and azalea had inhibition activity at low concentrations, whereas at high concentrations, they acted as a growth bio-stimulant. Comfrey and azalea at concentrations of 25−100% produced no phytotoxicity activity on red spinach seeds, whereas at 25− 50% concentrations, they had an inhibitory effect on hypocotyl and cotyledon growth parameters, while at 100%, they had a stimulatory effect on radicle, cotyledon and biomass parameters. Overall, the current bioactivity research showed that both the tested plants were active as inhibitors and biostimulants, supporting their development and utilization as bioherbicides and plant growth regulators through further research. In addition, research is necessary to investigate the inhibitory and stimulatory effects and allelochemical compound profiling of comfrey and azalea.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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