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Allelopathic effects of weed spp on the root border cells of *Fagopyrum tataricum* (L.) Gaertn

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ABSTRACT

We studied the allelopathic effects of aqueous extracts of *Galinsoga parviflora* Cav., *Chenopodium ambrosioides* L., and *Bidens pilosa* L. weed species on the developmental characteristics of Tartary buckwheat RBCs. The increase in the concentration of aqueous extracts, there was increase in apoptosis rate and mucigel thickness of the RBCs, level of intracellular reactive oxygen species and nitric oxide, but decreased the mitochondrial membrane potential ($P < 0.05$). The allelopathic effects of aqueous extracts of donor weed species on RBCs followed the order: *C. ambrosioides* > *B. pilosa* > *G. parviflora*. The increased levels of reactive oxygen species, nitric oxide and the reduced potential of mitochondrial membrane, further disrupted the homeostasis and normal metabolism of cells, thereby inducing the cell death. Among the three weed species, *C. ambrosioides* had the most significant impact.

Key words: Allelopathy, apoptosis mechanism, *Bidens pilosa, Chenopodium ambrosioides, Fagopyrum tataricum, Galinsoga parviflora,* oxidative damage, RBC, root border cells, Tartary buckwheat

INTRODUCTION

Tartary buckwheat [*Fagopyrum tataricum* (L.) Gaertn] is annual crop, rich in nutrients and bioactive elements (rutin, polyphenols, protein, polysaccharides, dietary fibre and lipids) (37, 44). It cures the hypertension, coronary heart disease, diabetes, etc (16) and used as food, drinks and cosmetics (23). The Sichuan Liangshan Yi Autonomous Prefecture is the place of orgin of Tartary buckwheat and produces largest quantity of Tartary buckwheat worldwide (42). However, its planting area is severely affected by invasive weeds viz., *Galinsoga parviflora* Cav, *Chenopodium ambrosioides* L., and *Bidens pilosa* L. severely reduces its yields (27). Their strong allelopathic effects decreased the Tartary buckwheat yields (27). According to the "novel weapon" hypothesis, allelopathy is one of the mechanisms for the successful invasion of exotic plants (2). Invasive plants release allelochemicals in the surrounding environment in many ways, inhibiting the growth and development of surrounding plants and thus establishing their dominance in an ecosystem. Many studies have shown that *G. parviflora* (20), *C. ambrosioides* (41) and *B. pilosa* (45) have strong allelopathic effects on crops.

The root tip is the most active part of root system. On the surface of root tip of most plants, there are highly metabolized root border cells (RBCs) that are different from cells of the root cap (15). These unique cells undergo active absorption and release of glucose and can survive in soil or water for several weeks or months (31). RBCs are the

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*Corresponding author. College of Life Science, Sichuan Normal University, Chengdu 610101, China. Mobile Number: +(86)137-0901-8761. E-mail address: danwei10ma@163.com, 549693288@qq.com protective barrier between the root and the soil environment (11) and provides resistance to environmental stress and protect the root tip tissues from bacteria, fungi, nematodes and metal ions (8). RBCs secrete proteins, extracellular DNA and other mucous substances to form a mucigel outside the cells, similar to that which occurs in neutrophil extracellular traps (6). The mucigel thickens under environmental stresses to trap external pathogens or chelated toxic substances, thus protecting the root system. For example, the mucigel secreted by RBCs of rice tightly binds and immobilize aluminium, which reduces its absorption by the roots and alleviates the inhibitory effects of aluminium on root growth (3). RBCs of pea (*Pisum sativum* L.) remove s the Pb from the lead solution (21). Allelochemicals released into the environment by plants in various ways eventually enters the soil (10). However, they must first break through the defensive line of RBCs to affect the roots activity and growth of plants. For example, allelochemicals such as p-hydroxybenzoic acid induced the RBCs of grapevine (*Vitis riparia* × *Vitis labrusca*) to secrete mucigel. The accumulation of salicylic acid in the cells activates the cellular antioxidant defensive system, which alleviates the stress caused by ρ-hydroxybenzoic acid (26). α-Terpinene and cymene decreases the activity of maize RBCs and pectin methylesterase; in contrast, the relative area of mucigel increased (18). However, there are few reports on the response and mechanism of RBCs of Tartary buckwheat to different malignant weeds in farmlands. We studied the changes in activity, mucigel thickness, mitochondrial membrane potential and reactive oxygen species (ROS) and nitric oxide (NO) levels of Tartary buckwheat RBCs treated with aqueous extracts of donor weed species. The test weeds used in these experiments were: *Galinsoga parviflora* Cav, *Chenopodium ambrosioides* L., and *Bidens pilosa* L., (common weed species in Tartary buckwheat growing region). This study aimed (i). to understand the defensive mechanism of Tartary buckwheat RBCs in response to weed allelopathy and (ii). to provide the theoretical basis of weed control in Tartary buckwheat fields.

MATERIALS AND METHODS

Plant Material

The seeds of Tartary buckwheat were collected in Mianning County, Sichuan Liangshan Yi Autonomous Prefecture, China (101°55′53.04″ E, 28°19′14.17″ N, 1980 m above sea level, annual rainfall.: 1150 mm, annual maximum temp.: 20 °C and a minimum temp.: 10 °C. The shoots of *G. parviflora, B. pilosa* and *C. ambrosioides* were collected at the flowering stage from Tartary buckwheat fields in Mianning County.

Preparation of Aqueous Extracts

The study was conducted from June 2019 to May 2020. The shoots of *G. parviflora*, *B. pilosa* and *C. ambrosioides* were dried in shade at 25 °C for two weeks and cut into 2-3 cm pieces. Thirty g cut pieces were soaked in 300 mL water (1: 10 ratio) in different Erlenmeyer flasks. These were shaken at 25 °C and 130 r/min for 48 h on shaker. Thereafter, the slurry was filtered twice through four layers of gauze to obtain pure aqueous extracts of 0.1 g.mL^{-1} conc. The aqueous extract of each weed species were sealed in brown bottles and stored at 4 °C for later use. During the tests, the pure extracts were diluted with distilled water to make 0.01, 0.02, 0.03, 0.04, and 0.05 g.mL^{-1} concentrations.

Agar Culture

Pure agar media AGAR powder was mixed with distilled water at 1:125 (0.8 %) and boiled, until AGAR powder was completely dissolved. AGAR was purchased from Guiben Instrument Co., Ltd., Chengdu, China. Thirty mL agar media was added in each glass culture flask (height 90 mm, diameter 68 mm, calibre 65 mm). The flasks with the media were sterilized at 121 °C for 20 min, thereafter cooled and solidified for use.

Tartary buckwheat seeds of uniform size were sterilized with 0.5 % $KMnO_4$ for 20 min and then rinsed with distilled water until the liquid was colourless. The seeds were then transferred to a beaker and soaked in distilled water for 24 h. About 2000 soaked seeds were transferred to a white porcelain dish (covered with gauze to minimize moisture loss) and it was placed in dark incubator at 25 °C for 24 h. After the radicles appeared, the 15-seeds were inserted into a glass culture flask containing 0.8 % agar media, with radicles outwards as shown in photograph 1. The lid of glass culture flask was screwed tightly, and flask was placed upside down in dark incubator at 25 °C. The radicle culture was based on the method of Chen *et al.* (7). These seedlings were randomly divided into two groups, ‘A’ and ‘B’ as under.

Photograph 1. Showing the method of inserting seedlings in Agar media

(i). Group A: These were used to determine the cell developmental characteristics of RBCs of buckwheat seedlings. The RBCs were isolated from the root tips when the radicle was 5, 10, 15, 20, 25, 30, and 35 mm long. The RBCs produced from the roots of 10 buckwheat seedlings were collected by immersing the root tips in 100 μL distilled water for 1-2 min and oscillating with a vortex oscillator for 30 s to release the RBCs. The root tips were removed with a tweezer to prepare RBC suspensions. The number, activity and mucigel thickness of RBCs were measured.

(ii). Group B: These were used for the allelopathic test. The RBCs were isolated from the root tips, when the radicle was 25 mm long. To ensure that the number of RBCs in each treatment were same, the RBCs produced from 60 radicles were collected by immersing the root tips in 600 μL distilled water and oscillating with a vortex oscillator for 30 s to release the RBCs. The root tips were removed, and the RBC suspension was shaken and divided into six equal parts. Each sample was centrifuged for 5 min at 1000 rpm at 4 °C, thereafter, the distilled water was discarded. The 100 μL of aqueous extract with different concentrations (0.01, 0.02, 0.03, 0.04 and 0.05 g $mL^{-1)}$ were added to each sample, while the control, received 100 μL distilled water. After 30 min, the activity, mucigel thickness, intracellular ROS, NO, and mitochondrial membrane potential of the RBCs were measured.

Root Boarder Cells (RBC)

(i). Numbers: A 20 μL RBC suspension was taken from each treatment in group A and group B and added to a blood cell counting plate. Ten μL of 4 % trypan blue was added for staining. The number of cells were counted under an Eclipse 55i light microscope (Nikon, Japan). Each treatment was repeated five times.

(ii). Activity: A 10 μL RBC suspension aliquot was taken from each treatment in group A and group B. They were placed in different centrifuge tubes. The RBCs were stained with 4 μL of 0.1 $mg.mL^{-1}$ AO/EB (AO, 3,6-bis(dimethylamino)acridine zinc chloride hydrochloride; EB, ethidium bromide) in dark for 2 min. The stained suspension cells (10 μL) were placed between a slide and coverslip and observed under blue excitation light of Nikon Eclipse 55i fluorescence microscope (Nikon, Japan) (28). A total of 1000 cells were counted, with apoptotic cells, necrotic cells and living cells appearing orange, red and green, respectively. Each treatment was repeated five times. The survival rate and apoptosis rate of the RBCs were calculated as under:

Survival rate (%) = Number of living cells/Total number of cells × 100%
Apoptosis rate (%) = Number of apoptotic cells/Total number of cells × 100%

(iii). Mucigel Thickness: A 10 μL RBC suspension aliquot was taken from each treatment in group A and group B. They were kept in different centrifuge tubes. The mucigel of RBCs was stained with 10 μL of Indian ink for 2 min (3). The stained RBC suspension (10 μL) was placed between slide and a coverslip and then observed. The mucigel thickness of three different positions of RBCs was measured under a Nikon Eclipse 55i light microscope. Each treatment was repeated ten times.

Reactive Oxygen Species (ROS)

The generation of ROS was determined by 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe. The samples in group B were centrifuged for 5 min at 1000 rpm at 4 °C, and aqueous extracts were discarded. RBCs were then rinsed with phosphate-buffered saline (PBS). DCFH-DA was diluted with PBS in ratio of 1:1000 to final concentration of 10 μM. The suspended RBCs were incubated in 10 μM DCFH-DA for 30 min at 37 °C in dark. Dye-loaded cells were centrifuged at 1000 rpm for

5 min, and the RBCs were washed with PBS several times to remove the excess fluorophore. The RBCs were subsequently re-suspended in 100 µL of PBS. The intracellular ROS production and distribution were visualized via blue excitation light of Nikon Eclipse 55i fluorescence microscope. The relative fluorescence intensity of DCF was measured with a Spectra Max M2 (Molecular Devices, USA) device at room temperature, with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Each treatment was repeated five times.

Nitric Oxide (NO)

The generation of NO was determined by 3-amino,4-aminomethyl-2’,7’-difluorescein diacetate (DAF-FM DA) fluorescent probe. The probe was loaded before RBCs were treated with aqueous extracts, to measure their NO fluorescence intensity. The suspended cells were incubated in 5 µM DAF-FM DA for 25 min at 37 °C in the dark. Dye-loaded cells were centrifuged at 1000 rpm for 5 min, after which the RBCs were washed with PBS several times to remove the excess fluorophore. Aqueous extracts were then added to each sample, while the control group was given the same amount of distilled water. After 30 min, all treatments were centrifuged for 5 min at 1000 rpm at 4 °C, thereafter the aqueous extracts were discarded. The RBCs were subsequently rinsed with PBS and resuspended in 100 µL of PBS. The intracellular NO production and distribution were visualized with the blue excitation light of Nikon Eclipse 55i fluorescence microscope. The relative fluorescence intensity of the DCF was measured with a Spectra Max M2 instrument at room temperature, with an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Each treatment was repeated five times.

Mitochondrial Membrane Potential ($\Delta\Psi m$)

The mitochondrial membrane potential of the RBCs was measured with a mitochondrial membrane potential detection kit from Beyotime (Chengdu, China). The samples in group B were centrifuged for 5 min at 1000 rpm at 4 °C, the aqueous extracts were discarded and the RBCs were rinsed with PBS. The suspended RBCs were incubated in 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) dye solution in dark for 20 min. The cells were then rinsed with JC-1 (1X) buffer solution thrice, after which the RBCs were re- suspended in 100 µL of JC-1 (1X) buffer solution. The JC-1 monomer and JC-1 aggregate fluorescence intensities were measured with Spectra Max M2 (Molecular Devices, USA) instrument at room temperature, an excitation wavelength of 490 nm and an emission wavelength of 530 nm for fluorescence of JC-1 monomers and with an excitation wavelength of 525 nm and an emission wavelength of 590 nm for the J aggregates. When the mitochondrial membrane potential is high, JC-1 accumulates in the mitochondrial matrix to form a polymer that produces red fluorescence. When the mitochondrial membrane potential was low, JC-1 does not accumulate in the mitochondrial matrix. In this case, JC-1 was a monomer and could produce green fluorescence. The relative ratio of red/green fluorescence was used to measure the proportion of mitochondrial depolarization (38). Each treatment was repeated five times.

Statistical Analysis

The allelopathic effect index (*RI*) was calculated asper Lin (25) as under:

$$RI = 1 - C/T (T \geq C)$$

$$RI = T/C - 1 (T < C)$$

Where, *C*: Control and *T*: Treatment. A *RI* >0: Weed aqueous extract has an allelopathic promoting effect on RBCs; in contrast, it has an inhibitory effect on RBCs. The higher the absolute value of *RI*, the stronger was allelopathic effect.

The allelopathic synthesis effect (*SE*) was calculated using the *RI* mean values of above measurements (apoptosis rate, mucigel thickness, ROS, NO, mitochondrial membrane potential of RBCs). The statistical analysis was done using the statistical package SPSS Version 20.0 for Windows (SPSS Inc., USA). The statistical significance of differences between the values of the different treatments was evaluated through one-way analysis of variance (ANOVA) with the least significant difference (LSD) post hoc test. P < 0.05 was considered to indicate a significant difference.

RESULTS AND DISCUSSION

Developmental characteristics of RBCs of Tartary buckwheat

As shown in Fig. 1, with the growth of Tartary buckwheat roots, the number of RBCs first increased and then decreased. When the length of the roots was 25 cm, the number of RBCs reached the maximum – 2315. The number of RBCs was decreased thereafter (P <0.05). With the growth of roots of Tartary buckwheat, the activity of RBCs reached the maximum (90.71 %) when the root length was 20 cm and the minimum (87.37 %) when the root length was 35 cm (P <0.05). There were no significant differences in RBC activity in the other groups (P >0.05), and they were approximately 90 %.

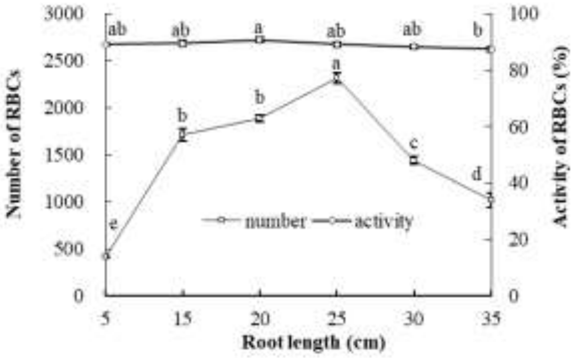

Figure 1. The number and activity of RBCs of *Fagopyrum tataricum* (L.) Gaertn varied with the length of the root. The different lowercase letters indicate significant differences at P <0.05.

Similarly, with the growth of the roots of Tartary buckwheat, the mucigel thickness of the RBCs first increased and then decreased (Fig. 2), with the maximum mucigel thickness (4 μm) at the root length of 30 cm and the minimum (5 μm) at the root length of 5 cm ($P < 0.05$).

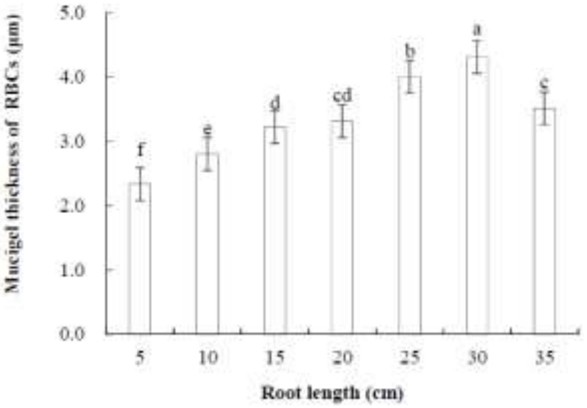

Figure 2. The mucigel thickness of RBCs of *Fagopyrum tataricum* L. varied with the length of the roots. The different lowercase letters indicate significant differences at $P < 0.05$.

When the root cap is in water, RBCs are released within seconds and the detached cells can still survive. This process is facilitated by pectin methylesterase and polygalacturonase, which are secreted by RBCs (35). The numbers of RBCs released daily from the root cap (0-10000 cells) are nearly constant. The number of RBCs released is dependent on plant species as there are significant differences in numbers between different species (15); however, there are no significant differences between the different plants of same species. For example, nearly 2,000 RBCs were released when the root caps of *Fagopyrum esculentum* Moench were immersed in water for 2~3 min, and their survival rate was 94 % when the root length was 25 mm (4). We found that the number, activity and mucigel thickness of RBCs of Tartary buckwheat changed with increased root growth: when the root length was 25 mm, the number of RBCs reached the maximum (2315 cells), their activity was about 90 % (Fig. 1), and their mucigel thickness was 4 μm (Fig. 2).

Apoptosis rate of RBCs

The increase in the concentration of aqueous extracts of three weed species resulted in consistent increase in the apoptosis rates of the RBCs (Fig. 3). The results showed that the activity the of RBCs decreased gradually, and there were significant differences among the treated concentrations ($P<0.05$). At 0.01 $g.mL^{-1}$, there were no significant differences in the apoptosis rate between the *G. parviflora* and *B. pilosa* treatment groups ($P<0.05$), while there were significant differences between the three weed species at the other treatment concentrations of the aqueous extracts ($P < 0.05$). The apoptosis rates of the RBCs in the *G. parviflora*, *B. pilosa* and *C. ambrosioides* treatment groups (0.05 $g.mL^{-1}$) increased by 431.17 %, 472.81 %, 480.88 %, respectively.

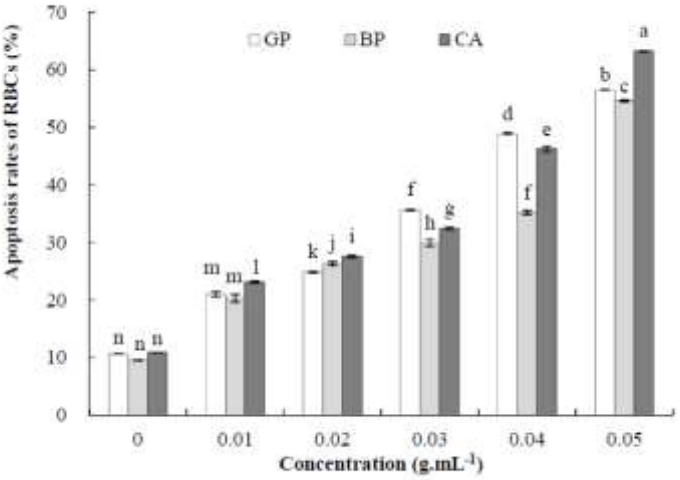

Figure 3. Effects of *G. parviflora* (GP), *B. pilosa* (BP) and *C. ambrosioides* (CA) aqueous extracts at different concentrations on the apoptosis rate of RBCs of *Fagopyrum tataricum* L. The different lowercase letters indicate significant differences (P <0.05).

Allelopathy is the release of allelochemicals from plants through leaching, volatilization, root exudation or residual decomposition, directly or indirectly affecting adjacent other plants or microorganisms (34). Under cinnamic acid stress, the apoptosis rate of RBCs of *Cucurbita moschata* is high, and their mucigel thickens (33). The extract of *Eupatorium adenophorum* increases the apoptosis rate of RBCs of *Amaranthus retroflexus*, *Echinochloa crus-galli*, and *Chenopodium glaucum*, damages the RBC protection system of root tips and ultimately inhibits the root growth and development (30). Under the stresses of coumarin and caffeic acid, the activity of RBCs of *Medicago sativa* decreases (39). Our results of this study were consistent with above studies. Some studies have shown that programmed cell death is the main mechanism of plants to resist stress, when they cannot adapt to endogenous and exogenous stresses (29). Our study showed that the RBCs of Tartary buckwheat resisted the allelochemicals and alleviated the damage of root tips through apoptosis in response to aqueous extracts of three weed species, especially the extracts of *C. ambrosioides* and *B pilosa*, caused the most damage to the RBCs of Tartary buckwheat (Fig 3).

Mucigel Thickness of RBCs

As shown in Fig 4 and 5, with a gradual increase in the concentration of aqueous extracts of the three weed species, the mucigel thickness of the RBCs of Tartary buckwheat became thicker, and there were significant differences between the treatment groups and the control group ($P<0.05$). The changes in mucigel thickness of the RBCs under the treatment of *G. parviflora* were more evident than those under the other two. At an extract concentration of 0.05 g.mL^{-1}, the mucigel thickness of the RBCs was 14.55 μm,

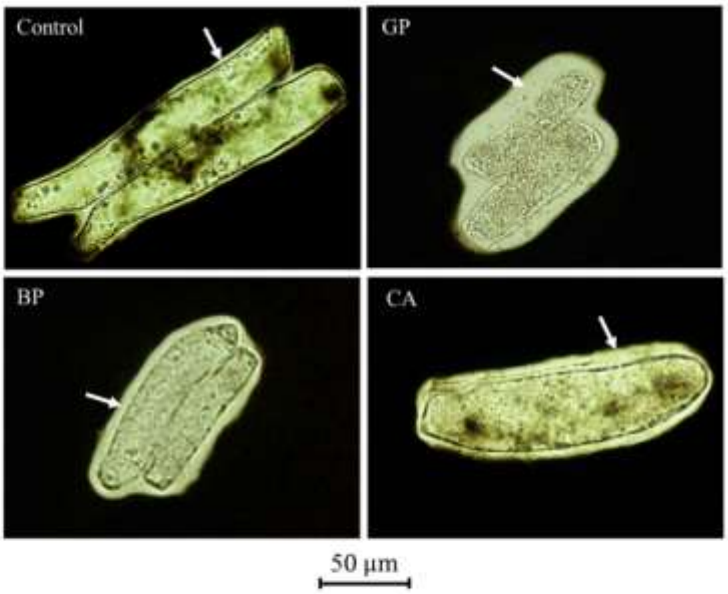

Figure 4. Effects of *G. parviflora* (GP), *B. pilosa* (BP) and *C. ambrosioides* (CA) aqueous extracts at 5 g.mL^{-1} on mucigel of RBCs of *Fagopyrum tataricum* (L.). Note: The white arrows point to the mucigel of RBCs.

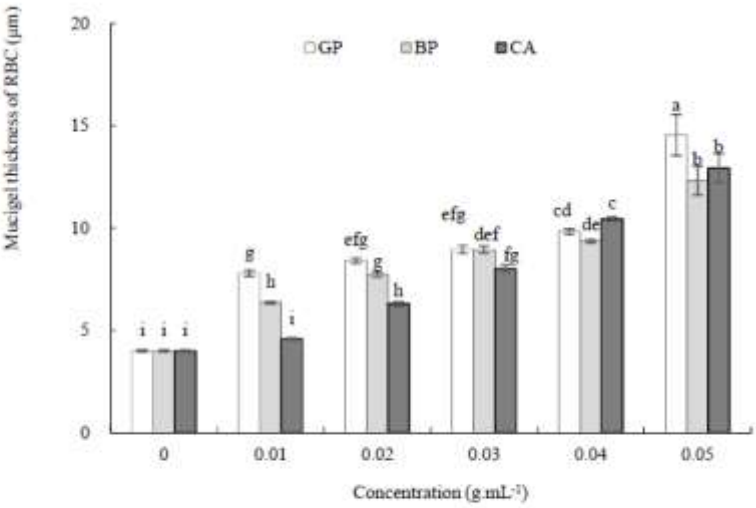

Figure 5. Effects of *G. parviflora* (GP), *B. pilosa* (BP) and *C. ambrosioides* (CA) aqueous extracts on the mucigel thickness of RBCs of *Fagopyrum tataricum* L. The different lowercase letters indicate significant differences (P <0.05).

12.32 μm, and 12.93 μm under the aqueous extracts of *G. parviflora*, *B. pilosa*, and *C. ambrosioides*, respectively. Combined with the results of the analysis of the apoptosis rate, the results showed that under the same treatment concentration, the mucigel thickness of the RBCs was the thickest, while the apoptosis rate was the lowest in the treatment group of the *G. parviflora* aqueous extract. However, the treatment group of *C. ambrosioides* was the opposite. This result indicated that the mucigel of RBCs alleviated the apoptosis induced by the aqueous extracts.

RBCs are rich in mitochondria, vacuoles, endoplasmic reticulum, Golgi bodies and secretory vesicles and exhibit intense metabolic activity (6). RBCs secrete extracellular proteins, exDNA, sugars and other substances outside, to form a mucigel around themselves. Polysaccharides can promote root caps to penetrate into the soil, and proteins can kill bacteria; control microbial cell division and act as attractants for bacteria, fungi and nematodes (22). DNA enzymes or proteases can degrade the exDNA or extracellular proteins in the mucigel of RBCs of maize, reducing the area of mucigel and decreases the activity of RBCs (18). The thickening of the mucigel of RBCs is a self-protection response of those cells to allelopathy. For example, under the stimulation of metal, mucigel secreted by RBCs chelates the metal ions to prevent the cells from absorbing the metal ions (32). When the relationships between the mucigel thickness and the apoptosis rate of RBCs in the three test weeds aqueous extract treatment groups were compared, the mucigel of RBCs was thickest and the apoptosis rate was lowest in *G. parviflora* aqueous extract. In contrast, the mucigel thickness of RBCs was thinnest, and the apoptosis rate was highest *C. ambrosioides* aqueous extract (Fig 3 and 5). Our results showed that, compared with the other two extracts, the *G. parviflora* aqueous extract was more likely to stimulate the RBCs of Tartary buckwheat, secreting a large amount of protective mucigel to alleviate the damage caused by allelochemicals. (Fig 4 and 5) The toxicity of *C. ambrosioides* aqueous extract on the RBCs of Tartary buckwheat was strongest, which affected the intracellular mucigel secretion system and the mucigel secreted by the RBCs was not enough to resist the allelochemicals from the *C. ambrosioides* aqueous extract, resulting in apoptosis.

ROS in RBCs

As shown in Fig 6 and 7, the green fluorescence intensity of the RBCs increased with increasing concentrations of the aqueous extracts of three weed species, indicating that all the aqueous extracts of three weed species could induce the production of ROS in the RBCs of Tartary buckwheat. The higher the concentration of the aqueous extracts was, the higher the ROS level, and there were significant differences between the concentrations ($P<0.05$). At 0.05 g.mL^{-1}, the relative fluorescence intensity of the ROS in the RBCs treated with *G. parviflora*, *B. pilosa*, and *C. ambrosioides* was 5.48, 5.92, and 4.82, respectively. Compared with high concentrations of *G. parviflora* and *C. ambrosioides* extracts, a high concentration of *B. pilosa* aqueous extract had a more significant effect on the ROS level of the RBCs of Tartary buckwheat. According to Pearson's correlation analysis, the relative fluorescence intensity of ROS and the apoptosis rate of the RBCs were significantly positively correlated under the treatment of aqueous extracts of three weed species ($P <0.01$), with correlation coefficient being 0.944 for *G. parviflora,* 0.975 for *B. pilosa* and 0.976 for *C. ambrosioides.*

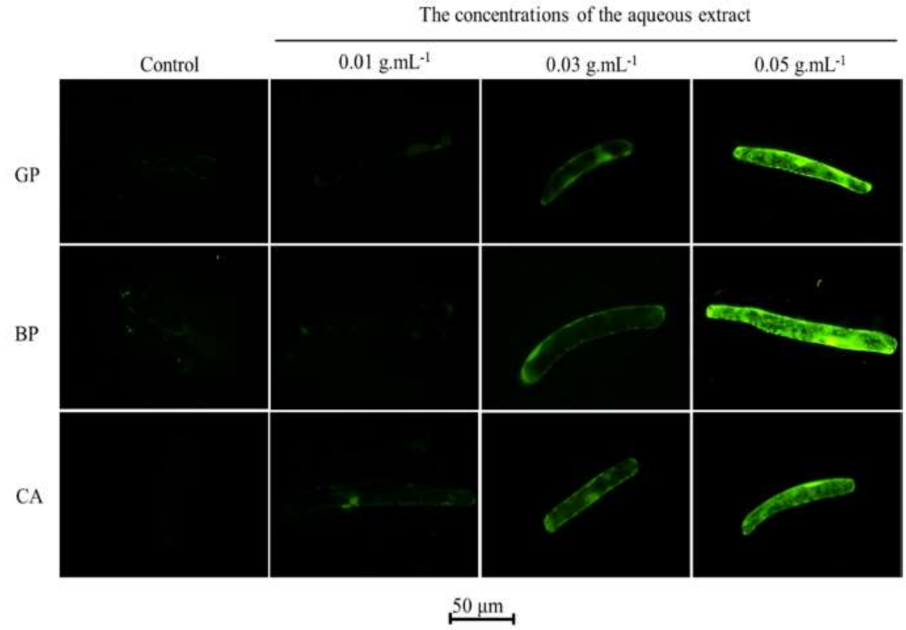

Figure 6. Localization of ROS in RBCs of *Fagopyrum tataricum* (L.) treated with aqueous extracts of *G. parviflora* (GP), *B. pilosa* (BP) and *C. ambrosioide*s (CA).

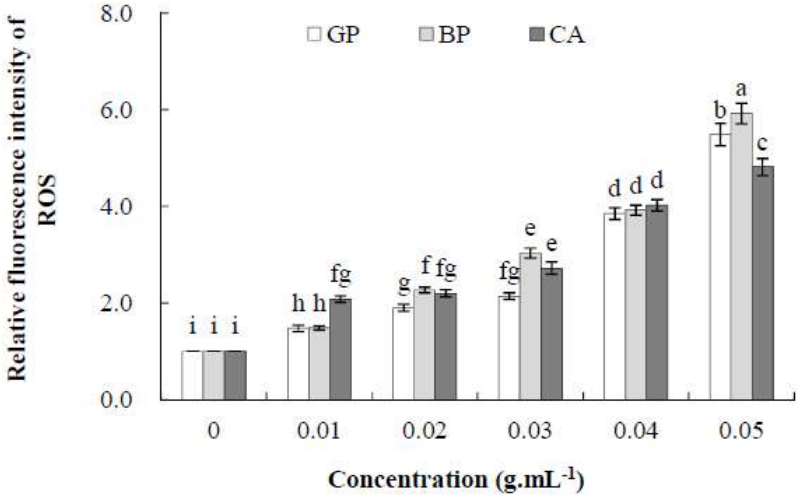

Figure 7. Change in ROS relative fluorescence intensities in RBCs of *Fagopyrum tataricum* (L.) treated with aqueous extracts of *G. parviflora* (GP), *B. pilosa* (BP) and *C. ambrosioides* (CA). The different lowercase letters indicate significant differences (P <0.05).

During the process of plant growth and development, biological or abiotic stresses (pathogens, heat and cold) cause cells to produce excess ROS, which accumulates in the cells. ROS can cause irreversible cell damage through their strong oxidative properties, which can disrupt the normal metabolic function of cells and alter the morphological structure of cells (19). Excessive accumulation of ROS affects the function of proteins, lipids and nucleic acids in cells, leading to cell damage and even death (13). Therefore, ROS are considered critical molecules in animal and plant cells in the study of apoptosis-related mechanisms. In our this study, the aqueous extracts of all three weed species induced ROS production in the RBCs and the level of ROS increased with increasing concentrations of aqueous extracts (Fig 6 and 7). According to Pearson's correlation analysis, there was a significant positive correlation between the apoptosis rate and ROS level of RBCs under treatment of three aqueous extracts. The results showed an ROS burst in the RBCs of Tartary buckwheat under the treatment of aqueous extracts of three weed species. RBCs were induced to produce more ROS in response to the aqueous extract of *C. ambrosioides* compared with other two species.

NO in RBCs

The green fluorescence intensity of NO in the RBCs increased significantly with increasing concentrations of the three aqueous weed extracts ($P<0.05$), indicating that all three aqueous extracts induced an increase in NO levels in the RBCs of Tartary buckwheat (Fig 8 and 9). The aqueous extract of *C. ambrosioides* had the greatest influence on the NO level in RBCs of Tartary buckwheat. Under the same treatment concentration, there were no significant differences in the NO level of RBCs in the treatment group of aqueous extracts of *G. parviflora* and *B. pilosa* ($P>0.05$). When RBCs were treated with the highest concentration of aqueous extracts ($0.05\ g.mL^{-1}$), the relative fluorescence NO values in the *G. parviflora, B. pilosa,* and *C. ambrosioides* groups were 1.33, 1.36 and 1.40, respectively. There were significant differences between the treatments of *G. parviflora* and *C. ambrosioides* ($P<0.05$). According to Pearson's correlation analysis, NO relative fluorescence intensity and apoptosis rate of the RBCs were significantly positively correlated under the treatment of the aqueous extracts of three weed species ($P<0.01$), and the species and their correlation coefficient were as follows: *G. parviflora* (0.953) > *B. pilosa* (0.919) > *C. ambrosioides* (0.857).

NO is involved in plants development (36) and in other important physiological reactions, such as cell death. NO is generated in plant cells under adverse conditions. For example, after short-term treatment with heavy metals, NO level increases and accumulates in most plant cells, which is inhibitory to growth of plant roots (40). When *Arabidopsis thaliana* is inoculated with non-toxic bacteria, a high concentration of NO is produced in plant cells (9). NO has dual effects on plant cells, its low concentration can promote cell resistance to stress. For example, the outbreak of NO in root tips of rice seedlings at the early stage of water stress enhanced the antioxidant ability of root tips and reduced the accumulation of ROS, which improved the adaptability of rice seedlings to water stress (5). However, high concentration of NO in plant cells, prevents the transfer of electrons in the electron transport chain to cytochrome C oxidase, resulting in an increase in hydrogen peroxide in mitochondria, is very toxic to cells (1). Some studies have shown

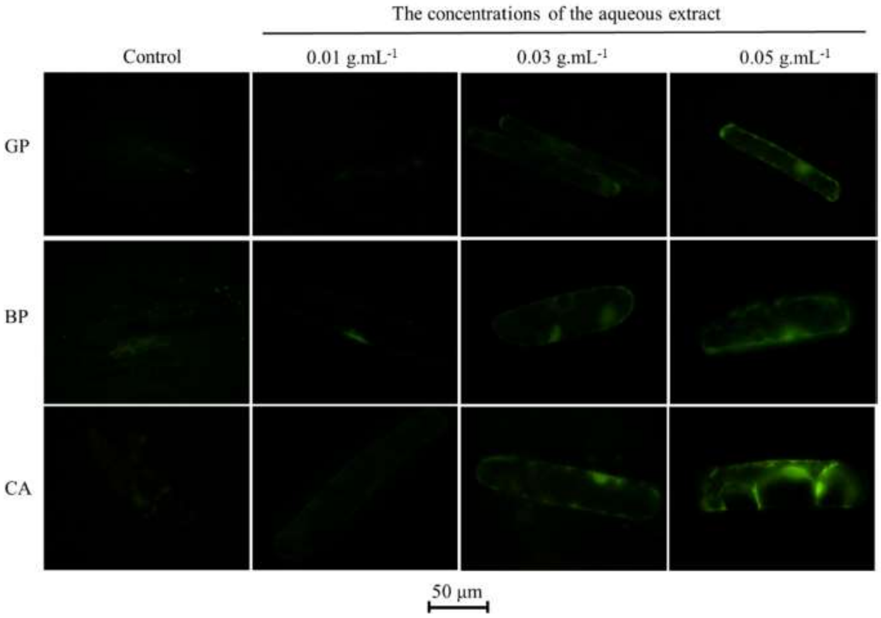

Figure 8. Localization of NO in RBCs of *Fagopyrum tataricum* (L.) treated with aqueous extracts of *G. parviflora* (GP), *B. pilosa* (BP) and *C. ambrosioides* (CA).

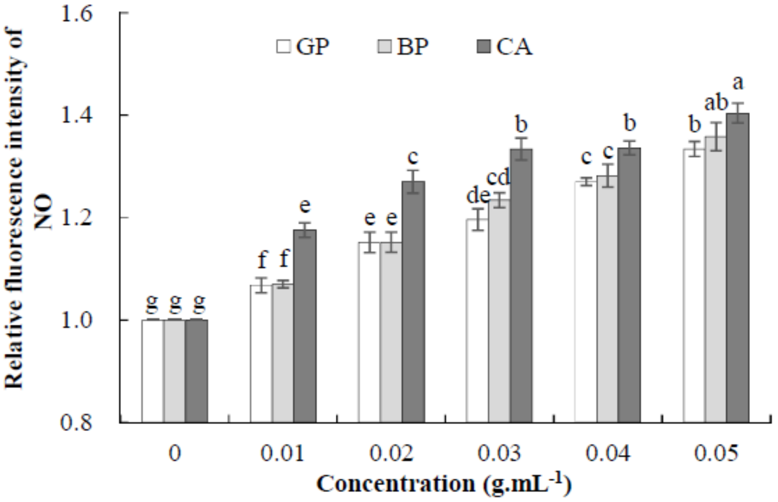

Figure 9. Changes in NO relative fluorescence intensities in RBCs of *Fagopyrum tataricum* (L.) treated with aqueous extracts of *G. parviflora* (GP), *B. pilosa* (BP) and *C. ambrosioides* (CA). The different lowercase letters indicate significant differences ($P < 0.05$).

that NO can affect the antioxidant system, leading to oxidative damage to plants, such as the increase in NO induced by SO_2 in *Tagetes erecta*, causing an ROS burst to regulate cell death (12). In the tyrosine nitrification mediated by peroxynitrite in *A. thaliana*, NO inhibits the activity of SOD and decreases the ability of cells to remove O_2^- (17). In the apoptotic state, cells are more likely to be oxidized, leading to the prolongation of NO life, thus activating the NO-mediated apoptosis signalling pathway (14). Our this study showed that the aqueous extracts of all three weed species induced RBCs of Tartary buckwheat to produce NO. With increasing concentrations of aqueous extracts, the level of NO increased significantly (Fig 8 and 9). The aqueous extracts of *C. ambrosioides* produced more NO, indicating that NO in RBCs was more sensitive to the aqueous extracts of *C. ambrosioides* than other two species (Fig 9). According to Pearson’s correlation analysis, the apoptosis rate of RBCs treated with the aqueous extracts of three weed species was positively correlated with the intracellular NO level. We hypothesized that NO produced in RBCs induced by aqueous extracts of the three weed species would affect the antioxidant system or hydrogen peroxide level, causing further oxidative damage to RBCs and eventually leading to apoptosis. The apoptosis rate had strong correlation with intracellular NO level under the treatment of the *G. parviflora* aqueous extract. It is possible that, under the treatment of *G. parviflora* aqueous extract, the RBCs of Tartary buckwheat were highly dependent on the NO-mediated apoptosis signalling pathway.

Mitochondrial membrane potential of RBCs

As shown in Fig. 10, with an increase in the concentration of the aqueous extracts of three weed species, the red/green fluorescence ratio of the mitochondria of the RBCs decreased gradually, indicating that the aqueous extracts of all three weed species could induce the mitochondrial membrane potential of RBCs to decrease and depolarize. When the concentration of aqueous extracts was 0.02 $g.mL^{-1}$ or 0.03 $g.mL^{-1}$, there were no significant differences in the effects of any of the three aqueous extracts on RBCs' mitochondrial membrane potential ($P> 0.05$). However, at the concentration of 0.05 g/mL^{-1}, the aqueous extracts of *G. parviflora*, *B. pilosa*, and *C. ambrosioides* decreased the red/green fluorescence ratio by 55.57 %, 44.6 %, 63.38 %, respectively, compared with the control groups. These results indicated that *C. ambrosioides* had the most significant influence on the mitochondrial membrane potential of the RBCs. According to Pearson’s correlation analysis, under the treatment of the aqueous extracts of the three weed species, the mitochondrial membrane potential and apoptosis rate of the RBCs were negatively correlated ($P<0.01$), and the species and their absolute value of Pearson's correlation coefficient were: *C. ambrosioides* (0.896) > *B. pilosa* (0.868) >*G. parviflora* (0.706).

The mitochondrion is an essential site of ROS production and plays a vital role in ROS-induced programmed cell death. ROS can activate the mitochondrial permeability transition pore (PPTP) and increase the mitochondrial permeability, leading to the rupture of mitochondrial membrane structure and decrease in mitochondrial membrane potential (43). In aluminium-induced apoptosis in *Arabidopsis thaliana* mesophyll cells, the direct interaction between Al and FeS protein complexes in the mitochondrial respiratory chain lead to damage to complexes I and III, which leads to mitochondrial ROS bursts and the

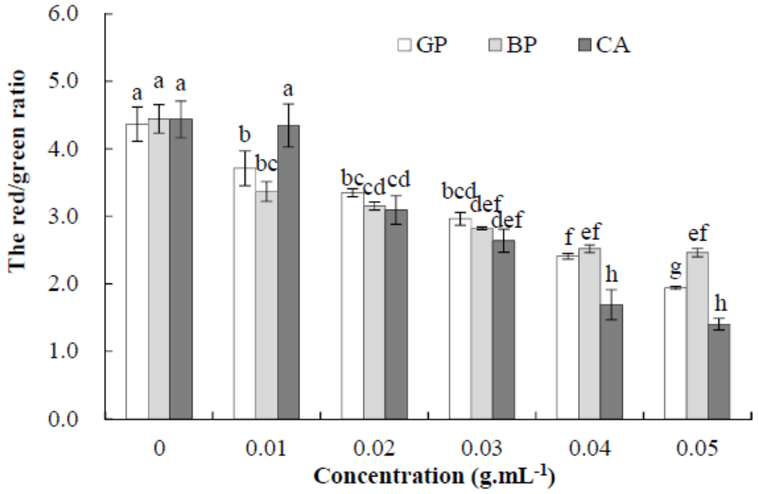

Figure 10. The red/green fluorescence ratio of the mitochondria of RBCs of *Fagopyrum tataricum* (L.) under the treatment of aqueous extracts of *G. parviflora* (GP), *B. pilosa* (BP) and *C. ambrosioides* (CA). The different lowercase letters indicate significant differences (P<0.05).

Table 1. Allelopathic and synthetic allelopathic effects of aqueous extracts of *G. parviflora*, *B. pilosa* and *C. ambrosioides* on RBCs of *Fagopyrum tataricum* (L.).

Donor	*RI*					*SE*
	Apoptosis rate	Mucigel thickness	ROS	NO	$\Delta\Psi m$	
Galinsoga parviflora Cav	0.67	0.58	0.58	0.16	-0.34	0.47
Bidens pilosa L.	0.68	0.53	0.63	0.17	-0.35	0.47
Chenopodium ambrosioides L.	0.68	0.46	0.65	0.23	-0.41	0.49

ROS-dependent breakdown of mitochondrial membrane potential (24). Under copper stress, cucumber RBCs produce ROS rapidly in a short period, and the mitochondrial membrane potential decreases at the same time. Moreover, oxidative stress and mitochondrial dysfunction stimulates the apoptosis process of RBCs in cucumber (38). In our this study, the aqueous extracts of all three weed species induced ROS production in the RBCs, and the level of ROS increased with increasing concentrations of aqueous extracts (Fig 6 and 7). However, the mitochondrial membrane potential decreased with increasing concentrations of aqueous extracts (Fig 10). Our results showed that aqueous extracts of the three weed species caused ROS burst in the RBCs of Tartary buckwheat, resulting in mitochondrial damage and inducing apoptosis.

Comparison of allelopathic effects of aqueous extracts of three weed species

The aqueous extracts of *G. parviflora*, *B. pilosa* and C. *ambrosioides* increased the apoptosis rate, mucigel thickness, and intracellular ROS and NO levels of RBCs but decreased the mitochondrial membrane potential (Table 1). According to the allelopathic synthesis effect (SE), the allelopathic effect of C. *ambrosioides* was the strongest on the RBCs of Tartary buckwheat among the three species.

CONCLUSIONS

The water soluble allelochemicals from weed species (*Bidens pilosa, Chenopodium ambrosioides, Galinsoga parviflora*) growing in Tartary buckwheat fields increased the ROS and NO levels and decreased the mitochondrial membrane potential of RBCs of buckwheat, which led to oxidative damage to RBCs and their apoptosis. Thus, allelopathic effects of these weed species on Tartary buckwheat, interfered with the defensive functions of recipient plants, resulting in disruption of growth and development of buckwheat plants. The *C. ambrosioides* RBCs were very allelopathic to apoptosis rate, mucigel thickness, ROS level, NO level, and mitochondrial membrane potential of buckwheat RBCs.

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