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Article

Elevated CO_2 Concentrations and Water Stress Affect the Ability of Italian Ryegrass to Remediate Herbicides and Enhance its Allelopathic Effect

Elevada Concentração de CO_2 Atmosférico e Déficit Hídrico Afetam a Habilidade do Azevém em Remediar Herbicidas do Solo e Aumentam Seu Potencial Alelopático

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ABSTRACT - The long temporal persistence of select herbicides negatively impacts crops sown in succession to irrigated rice. One way to reduce these compounds in the soil over time is through phytoremediation. However, elevated CO_2 concentrations may interfere with the phytoremediation process. Another consequence of climate change is the production of allelopathic compounds by forage species used as remedial agents. This study aimed to evaluate the impact of elevated CO_2 concentration and drought stress on the remediation of soil samples contaminated with imazapyr + imazapic herbicides by Italian ryegrass and any subsequential affect on the allelopathic effect of this species. We report that the increasing CO_2 decreased the phytoremediation potential of ryegrass. Water stress combined with a CO_2 concentration of 700 µmol mol^{-1} caused increased allelopathy. Overall, these are the first data to indicate a significant effect of higher CO_2 levels with respect to both phytoremediation efficacy and allelopathic potential of the plant species used in phytoremediation.

Keywords: climate change, *Lolium multiflorum* L., remediation, and allelopathy.

RESUMO - O residual prolongado dos herbicidas do grupo das imidazolinonas é um problema para as culturas semeadas em sucessão ao arroz irrigado. Uma maneira de reduzir a concentração destes compostos no solo é utilizando a fitorremediação. Contudo, o aumento da concentração de CO_2, pode interferir no processo de fitorremediação de plantas. Outra consequência que pode ser causada pelas mudanças climáticas é a produção de compostos alelopáticos das espécies forrageiras utilizadas como remediadoras. O presente estudo teve por objetivos quantificar se há perda no capacidade do azevém semeado em solo com residual de imazapir+imazapic de remediado quando submetido ao aumento da concentração de CO_2 na atmosfera, combinada com estresse hídrico e alta população. A combinação do estresse hídrico com a concentração de CO_2 de 700 µmol mol^{-1} causa aumento da alelopatia do azevém. De maneira geral, esses são os primeiros resultados que indicam o efeito significativo do aumento dos níveis de CO_2 com relação à eficácia de remediação e de alelopatia de plantas usadas para fitorremediação.

Palavras-chave: mudanças climáticas, *Lolium multiflorum* L., remediação, alelopatia.

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INTRODUCTION

Used as a tool to control weedy rice, the Clearfield™ system correspond to around 90% of the rice area on the 2016/17 harvest season for the state of Rio Grande do Sul (RS). In this system it is used imidazolinones herbicides to control weedy rice. Depending on the soil and environmental conditions, these herbicides may persist in the soil after the harvest (Kraemer et al., 2009). Rice soils in Rio Grande do Sul are predominantly acidic (Boeni et al., 2010), which favors the adsorption and retention of the imidazolinones (Kraemer et al., 2009) reducing herbicide dissipation and enhancing its persistence carryover to the next growing season. Herbicide persistence in turn may result in growth retardation and development of non-tolerant crops that follow Clearfield® rice (Sartori et al., 2011).

In RS state Italian ryegrass (*Lolium multiflorum* Lam.) is planted following Clearfield irrigated rice harvest (Pinto et al., 2009) due to its adaptation to poor drainage soils and its potential for use as pasture or cover crop (Menezes et al., 2001). Its use may suppress weed growth in the fallow, both physically (due to shading) and/or chemically, due to the release of allelopathic compounds that may retard or inhibit the development of other plants.

Ryegrass can also act as a phytorremediator, decreasing the concentration of herbicide contamination in the soil (Souto et al., 2013; Galon et al., 2014). This process can occur through absorption by the plants and further metabolism or in some cases are due to stimulation of the soil microbiota associated with the remediation plants.

Although the role of Italian ryegrass and other cover crops to act as phytoremediators for herbicide residual is well recognized, their efficacy may be impacted by climate change. These impacts may be associated with changes in temperature and precipitation (Miraglia et al., 2009; Delcour et al., 2015); or the direct stimulation of plant growth, altered phenology and nutritional uptake by rising CO2 levels (Mayers et al., 2014) and may affect sensitivity of weeds to herbicides (Refatti et al. 2019). However, at present the role of rising CO_2 on phytoremediation efficacy for cover crops such as Italian ryegrass is unknown.

To address this issue, the goal of the current study was to assess whether projected increases in elevated CO_2 altered the remediation of soil contaminated with imazapyr + imazapic herbicides using Italian ryegrass as the cover crop species. A secondary objective was to investigate the allelopathic effect of ryegrass when exposed to an increase in CO_2 concentration in the atmosphere, combined with water stress and high population density.

MATERIALS AND METHODS

Two experiments were conducted in open-top chambers (OTCs) at Federal University of Pelotas, Capão do Leão, Brazil. The OTC's measuring 1.90 x 1.90 x 2.00m (width/depth/height) with 3.6 m² of internal area. The levels of CO_2 were controlled by an automatic system consisting of sensors, processor and valves. This system centralizes information on a single processor capable to control CO_2 concentration and record data. The processor was programmed to collect CO_2 sensor readings every 10 sec. The data from each CO_2 sensor were stored in the internal memory of the processor for subsequent calculation of the amount of CO_2 to be added to the OTCs. The CO2 levels were automatically maintained by comparing the desired concentration programmed for each chamber with the CO_2 sensor reading. To satisfy the prescribed conditions, the amount of CO_2 to be injected into each OTC was calculated using a mathematical model established in previous experiments.

In the first experiment, ryegrass remediation capacity was evaluated at different concentrations of CO_2. In the second experiment, the allelopathic potential of ryegrass was tested at different CO_2 concentrations, soil moisture, and plant population levels. The soil (Albaqualf) used in the experiments was collected at 0 to 20 cm depth, in an area with no history of herbicide applications, and after seaving it was placed in a 4-liter volume pots (experimental unities).

The role of enhanced CO_2 on herbicide remediation was estimated in a bioassay. The experiment was conducted in a completely randomized experimental design with four replicates in a factorial arrangement (2 x 4). Factor A included two CO_2 concentrations inside the OTCs, 400 ± 50 (current) and 700 ± 50 μmol mol^{-1} (climate change scenario) (IPCC, 2014). Factor B included four rates of the herbicide imazapyr + imazapic: 0, 21, 70 and 140 g ha^{-1}, which correspond to 0, 15, 50 and 100% of the recommended rate, respectively (73.5 g $i.a.^{-1}$ + 24.5 g $i.a.^{-1}$). The timeline of the experiment is shown in Figure 1. At 24 hours after application, herbicide and soil was mixed and returned to the pots. The ryegrass was then sown and kept in the OTCs for 60 days. Three days after ryegrass removal, pots were then removed from OTCs and received seeds of the bioindicator rice cultivar (IRGA 424), which is sensitive to imidazolinones. The rice was grown under these conditions (ambient CO_2 concentration) for 40 days in a greenhouse. Finally, the ryegrass dry matter mass (DMM) and the rice plants height and visual injury were evaluated.

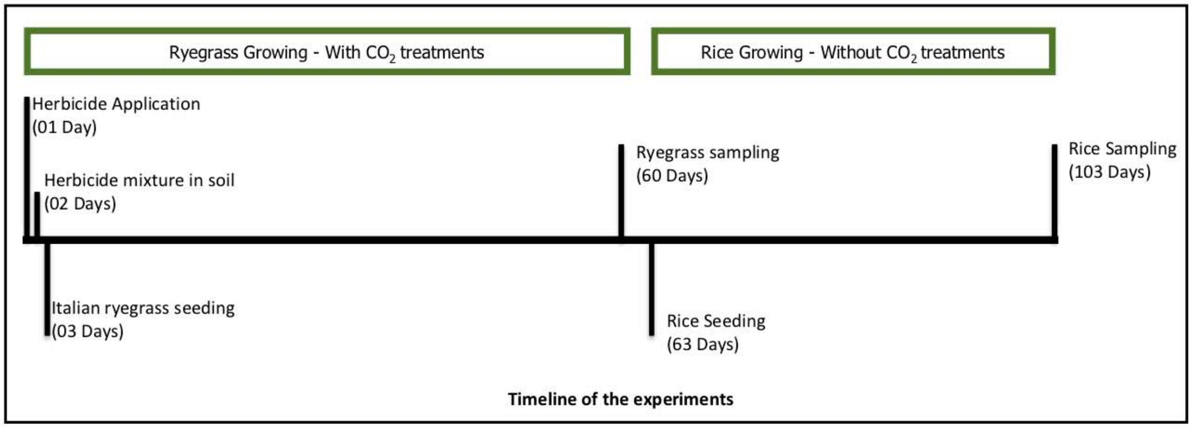


Figure 1 **-** Timeline of the remediation experiment with Italian ryegrass using soil samples with residues of the herbicides and different concentrations of CO_2, followed by residue testing with herbicide-sensitive rice plants.

The second experiment was conducted in a completely randomized experimental design with four replicates in a three-factorial arrangement (2x2x2); The Factor A included two CO_2 concentrations (400 and 700 μmol mol^{-1}), the Factor B included two levels of soil water stress [-10 kPa (field capacity) and -80 kPa water deficit] and the Factor C included two sowing density (ten and 20 plants per pot). The ryegrass was harvested 60 days after emergence, when it reached the flowering stage.

The aboveground parts of the ryegrass plants were collected and dried in a forced circulation oven at 40 °C for three days, to avoid volatilization of the allelopathic compounds. When the material was dry, it was cut into 1 cm length pieces. The extracts were prepared submersing plants in a proportion of 10% w/v of distilled for 24 hours in closed plastic pots, at room temperature, in the dark. The extract was then run through a vacuum filter and diluted in 2:1 distilled water. The pH of the extracts ranged from 5.9 to 6.5.

The bioindicator species used in the second experiment was lettuce (*Lactuca sativa*) cultivar Veneranda. The assay was carried out in Petri dishes with two sheets of germination tests. The petri dish corresponds to the experimental unity and it was repeated four times. In each Petri Dish it was placed 50 seeds of lettuce and the extract was added in the proportion of 2.5 times the weight of the paper (2 mL). Distilled water was used as the control treatment. The Petri dishes were kept in a BOD incubator with a photoperiod of 12h/12h light/dark and the constant temperature of 25 °C.

The number of germinated seeds was recorded daily for seven days to determine the speed of germination index (*S*). *S* was calculated based on the formula described by Wardle et al. (1991):

$$S = [(N1/1) + (N2/2) + (N3/3) + \dots + (Nn/n)]$$

where $N1$, $N2$, $N3$, and Nn are the number of germinated seeds and 1,2,3 … n, are the number of days after sowing.

After seven days, the number of total germinated seeds (%G), the length of aerial parts (LAP), of the root (LR), dry matter mass of the shoot (DMMS) and the root system (DMMR) were evaluated.

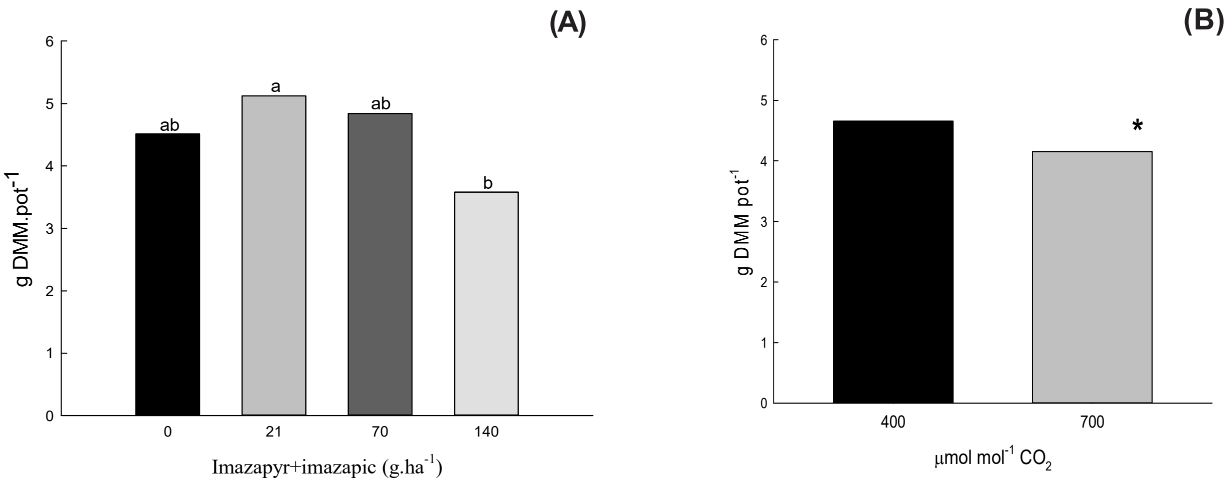
Lettuce length and dry mass measurements were taken from 15 plants from each experimental unit.

Data were analyzed for homoscedasticity and normality before they were submitted to analysis of variance ($p \leq 0.05$). When significant differences were found, the treatments were compared using the Tukey's test ($p \leq 0.05$). In case of significant differences between rates, or interaction between factors, an analysis was performed employing regression models ($p \leq 0.05$).

RESULTS AND DISCUSSION

Phytoremediation

To our knowledge this is the first assessment regarding the impact of projected CO_2 increases on phytoremediation efficacy for Italian ryegrass. There was an effect of both herbicide rates and CO_2 concentration on ryegrass DMMS but there was no interactions between the two factors (Figure 2). The plants treated with 21, 70 and 140 g ha^{-1} of herbicide (Figure 2A) did not differ in DMMS from the herbicide-free control indicating that herbicide tolerance of Italian ryegrass across a range of concentrations. For CO_2 (Figure 2B), the plants grown under higher concentration (700 µmol mol^{-1}) presented a lower DMMS in comparison to those grown at current environmental conditions (400 µmol mol^{-1}).



Different letters indicate significant differences between mean values according to the F test ($p \leq 0.05$).

Figure 2 - Mass of dry matter for aerial parts of Italian ryegrass relative to herbicide rate (A) and CO_2 concentrations (B).

The efficacy of herbicide remediation in soil with plants has been well demonstrated (Lewis et al., 2015; Lv et al., 2016). The current results with Italian ryegrass are consistent with these previous studies; i.e., the DMMS of the plants exposed to 200 g ha^{-1} herbicide did not significantly differ from herbicide-free plants (Souto et al., 2015). However, ryegrass tolerance has been known to vary. For example, ryegrass exposed to 140 g ha^{-1} of imazapyr + imazapic in pre-emergence showed 80% injury, relative to the control. Similarly, a reduction of 70% in ryegrass DMMS has been observed previously relative to the control (Pinto et al., 2009). Chemical control of several Italian ryegrass ecotypes with imazethapyr + imazapyr was close to 90% at the tenth day following application, indicating high herbicide efficacy (Clemmer et al., 2004).

To determine CO_2-influenced changes in the remediation effects of ryegrass, rice plants that were susceptible to the herbicide imazapyr + imazapic were planted in the same pots. Visual injury (Figure 3) indicated a significant interaction between herbicide rates and CO_2 concentration; specifically that injury symptoms were higher in rice plants grown at 700 µmol mol^{-1} CO_2 with 100% of the herbicide.

These effects were visible at 7 DAE, with rice grown at ambient CO_2 conditions indicating low visual injury (around 5%) even at the highest rate of the herbicide (Figure 3A); in contrast,

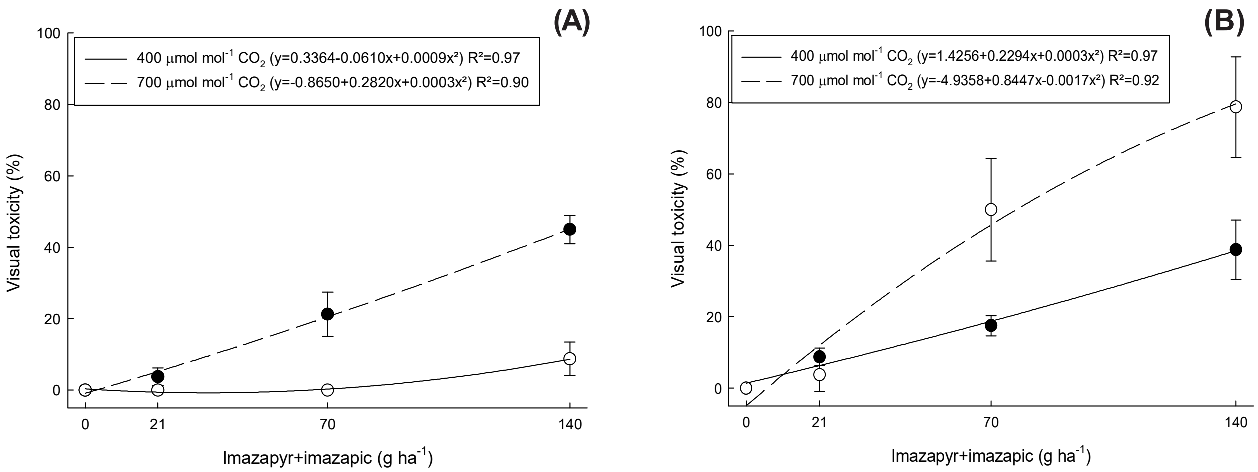
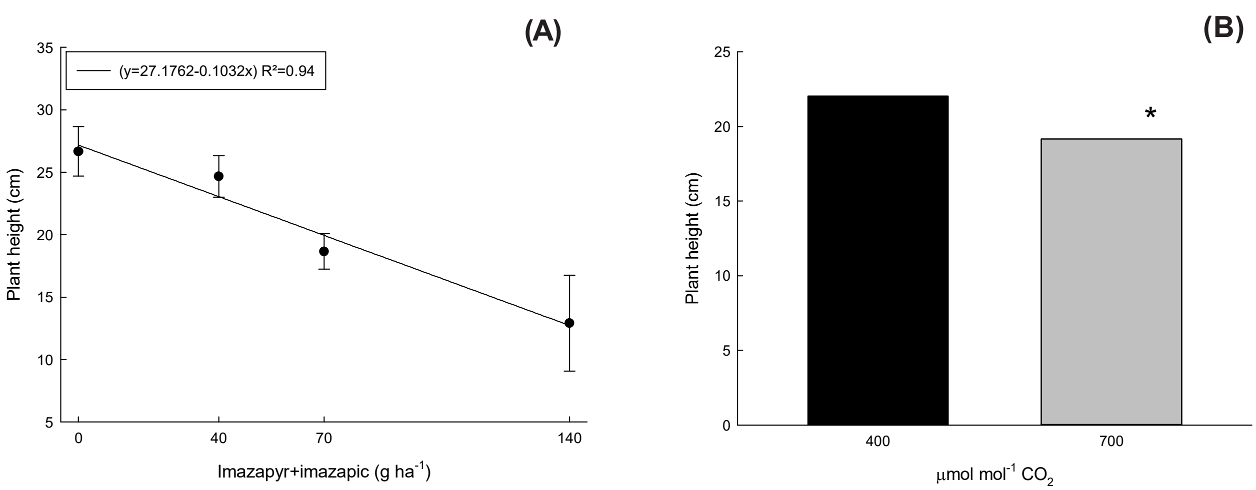


Figure 3 - Visual injury (%) observed in rice plants sowed after ryegrass exposed to different rates of imazapyr + imazapic herbicide and atmosphere CO_2 concentrations in 7 DAA (A) and 28 DAA (B).

visual injury on rice plants observed at 700 μmol mol^{-1} reached 40% at the highest rate (140 g ha^{-1}). By 28 DAE (Figure 3B), rice at 700 μmol mol^{-1} CO_2 reached about 80% visual injury, while those kept at 400 μmol mol^{-1} CO_2 reached 40% visual injury compared to the control.

Imidazolinones herbicides are an often-used means to chemically control red rice, but may also adversely impact sensitive rice lines grown in rotation (Marchesan et al., 2011; Refatti et al., 2017). Previous studies have indicated that the spraying of imazethapyr + imazapic and imazapyr + imazapic at recommended rates can promote visual injury at 21 days after emergence in sensitive rice plants (Refatti et al., 2017). These toxic effects also translate to concomitant changes in yield of sensitive rice (Marchesan et al., 2011). In addition, some studies have shown that ryegrass may have an allelopathic effect on rice biotypes, both cultivated and wild (Soares, 1991; Menezes et al., 2001). For example, rice cultivated in succession to ryegrass had lower rates of emergence and productivity compared to the control (Menezes et al., 2001).

For the current study, no interaction between factors was observed for the variable height of rice plants (Figure 4), but there were differences between the treatments of each factor. The mean herbicide rate in the soil adjusted to the curve (Figure 4A). The lowest height of plants was observed with the higher rate of herbicide. No statistical difference was observed when comparing the control plants (data not shown), which suggests the decrease in plant height was due to the presence of the herbicide and that there was no allelopathic effect of ryegrass in soil.



* Indicates a significant difference between mean values based on F test ($p \leq 0.05$).

Figure 4 - Plant height (cm) for irrigated rice plants evaluated 40 days after emergence in succession to the Italian ryegrass submitted to different rates of imazapyr + imazapic herbicide (A), and at different CO_2 concentrations in the atmosphere (B).

Rice plant height differed as a function of CO_2, with a significant decline associated with the elevated CO_2 concentration (Figure 4B). These data suggest that ryegrass, while tolerant to the sprayed herbicide may have been less effective on herbicide remediation at elevated CO_2 concentration, with subsequent effects on rice height. Alternatively, high CO_2 concentrations may have enhanced the production of allelochemicals.

With respect to remediation, although specific information on herbicide removal is not available, previous data by Guo et al. (2006) and Jia et al. (2007), indicated that elevated CO_2 could reduce heavy metal uptake in rice. Li et al. (2010) also concluded that at high atmospheric concentrations of CO_2, each rice cultivar would behave differently regarding the extraction of contaminating molecules from the soil.

CO_2 and water deficit effect on allelopathy

Italian ryegrass extracts influenced the speed of germination index (*S*) of lettuce seeds at concentrations of 5% and 10% (Tables 1 and 2). At 5%, significant effects were observed for CO_2 concentration and soil moisture, but interactions were not significant (Table 1). For soil moisture, *S* was 35% lower in water stress conditions, relative to field capacity. For CO_2 concentrations, the lowest *S* values were observed at elevated relative to ambient CO2 (~34% lower). In the extract at 10%, interactions between CO_2 concentration and soil moisture were observed for S (Table 2). At low soil moisture, CO_2 level does not alter allelopathic effect ryegrass; however, at field capacity, elevated CO_2 increased germination inhibition of lettuce seeds, reducing *S* by approximately 70%.

The percentage of germination of lettuce seeds (Tables 1 and 2) reflected a decrease in germination rates for all extracts when compared to the control treatment. Extracts at 5% and 10% reduced germination by 30 and 90%, respectively. However, no interaction was observed in treatments with the 5% concentration extract (Table 1), although there were effect of soil moisture and CO_2 concentration. Lettuce seeds exposed to the aqueous extract obtained from ryegrass plants submitted to field capacity and the current concentration of CO_2 presented the highest rates of germination.

Table 1 - Speed of germination index (*S*) and germination rate (%G) for lettuce seeds submitted to germination tests with aqueous extract of Italian ryegrass exposed to different CO_2 concentrations in the atmosphere, plant density, and soil moisture (kPa) at a 5% concentration

Water tension in the soil	*S*	% G
-10 kPa	51.39 a	92.50 a
-80 kPa	33.47 b	77.37 b
$[CO_2]$ in the atmosphere		
400 µmol mol^{-1}	51.32 a	95.38 a
700 µmol mol^{-1}	33.53 b	74.50 b
CV(%)	15.4	53.0

Means followed by the same lowercase letter in the column did not differ statistically from each other by the Tukey test ($p<0.05$).

Seed germination at the 10% concentration extract (Table 2) had interaction between soil moisture and CO_2 concentration. At elevated CO_2, soil moisture had no effect on germination rates. However, at 400 µmol mol^{-1} CO_2, ryegrass plants produced an allelopathic effect, reducing seed germination from 63% to 11% at field capacity and water deficit conditions, respectively. CO_2 concentration did result in a significant decrease in germination, ~54% between elevated and ambient conditions.

Table 2 - Speed of germination index (*S*) and germination rate (%G) for lettuce seeds submitted to germination tests with aqueous extract of Italian ryegrass exposed to different CO_2 concentrations in the atmosphere, plant density, and soil moisture (kPa) at a 10% concentration

Water tension in the soil	*S*		%G	
	400 µmol mol^{-1}	700 µmol mol^{-1}	400 µmol mol^{-1}	700 µmol mol^{-1}
-10 kPa	29.61 aA	9.88 aB	62.75 aA	28.50 aB
-80 kPa	3.23 bA	7.55 aA	10.75 bA	27.00 aB
CV (%)	23.6		65.0	

Lowercase letters refer to the comparison in the column, and uppercase letters refer to the comparison in the row according to Tukey's test ($p<0.5$).

Environmental factors such as light, water and temperature can alter alleopathic production and impact (Olofsdotter, et al., 2002), but at present the role of CO_2 on this process remains to be elucidated (Wang et al., 2009, 2010). In 750 µmol mol^{-1} of CO_2 increased the production of allelochemicals in *Mikania micrantha*, increasing injury on *Brassica campestres*, *Raphanus sativus* and *Lactuca sativa* (Wang et al., 2010). However, the ryegrass extract harvested in the field and harvested at the stem elongation phase did not affect the germination of lettuce and cucumber seeds (Castagnara et al., 2012; Bulegon et al., 2015).

The data from the present study indicate that a high concentration of CO_2 in the atmosphere impairs the remediation of imidazolinones by ryegrass, and secondly, that the high concentration of CO_2 combined with water deficit increases the allelopathic effect of ryegrass on germination and emergence speed of lettuce seeds. Additional studies should be conducted in order to quantify how much and why Italian ryegrass reduce the remediation potential. In addition, it is necessary to determine which compound causes the allelopathic effect and how it is affected by abiotic factors.

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