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Article

Enhancing trefoil germination under metal stress through *Bradyrhizobium* sp. (Lotus) inoculation

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Abstract

This study investigates the effect of inoculation with *Bradyrhizobium* sp. (Lotus), a symbiotic rhizobacterium, on the germination of trefoil under stress induced by copper, cadmium, and their combination. The goal is to highlight the role of *Bradyrhizobium* sp. (Lotus) in mitigating copper and cadmium stress and enhancing the germination of trefoil, a spontaneous fodder species. The research, conducted in Blida (Algeria) in 2024, focuses on the physiological aspects of germination, including Total Germination Count (TCG), Time to mean germination (TMG), and inhibition of germination reversibility. Statistical analysis was performed using Manova at a 5% threshold. Results indicate that *Bradyrhizobium* sp. (Lotus) inoculation increases TCG values and reduces the germination lag phase from +168 h to 48 h. Additionally, germination inhibition is decreased by 20 to 30% compared to non-inoculated seeds, and physiological germination parameters improve under metal stress conditions. The findings suggest that *Bradyrhizobium* sp. (Lotus) may mitigate copper and cadmium stress by detoxification or chelation, thereby enhancing trefoil tolerance to these metallic trace elements during the germination stage.

Keywords:

cadmium, copper, PGPR, physiology.

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Introduction

Trefoil holds significant ecological and agricultural importance due to its adaptability to various environments and its role as a forage legume. It contributes to soil fertility through biological nitrogen fixation, which enhances soil nutrient content and reduces the need for chemical fertilizers, making it an ideal candidate for sustainable agriculture (Smýkal *et al*., 2015). Its ability to thrive in nutrient-poor soils makes it particularly valuable in land restoration efforts (Brockwell *et al*., 2005).

Sustainable development initiatives worldwide increasingly emphasize the incompatibility of environmental pollution, particularly from persistent compounds like metallic trace elements, with ecological integrity. These elements, stable and persistent, pose significant contamination risks by accumulating in ecosystems and transferring to higher organisms, thereby impacting public health and ecological balance (Croteau *et al*., 2005; DeForest *et al*., 2007). Some metals exhibit toxicity even at low concentrations (Mills *et al*., 1977).

Microorganisms, particularly bacteria, play vital roles as builders, regulators, fixers, and stabilizers in the environment, such as atmospheric nitrogen fixation by rhizobacteria (Brockwell *et al*., 2005). Their diversity and activity can be significantly affected by various factors, including high concentrations of metal ions in soil, which induce structural, biochemical, and physiological changes in seeds, ultimately reducing germination rates and delaying plant development (Ashraf *et al*., 2007).

Despite numerous environmental constraints that can impede seed germination, few studies have explored the impact of metallic trace element stress on germination. The aim of this study is to evaluate the role of inoculating the rhizobacterium *Bradyrhizobium* sp. (Lotus) in mitigating trefoil seed germination impairment under copper stress.

Material and methods

Plant material

Seeds used in this experiment are those of *Lotus ornithopodioïdes* L., a spontaneous *Fabaceae* with a foraged character. *Lotus ornithopodioïdes* L. is wild distributed in the Mediterranean aera. Seeds are collected in Soumâa region (Blida-Algeria) in May 2023.

Bacterial strain

The bacterium used in this experiment is *Bradyrhizobium* sp. (Lotus) isolated from *Lotus ornithopodioïdes* L. nodules. It is used at a concentration of 10^8 CFU ml^{-1} aged 24 h (Degaichia *et al*., 2024).

Inoculation and germination essay

Sterilization of seeds is carried out according to the method of Vincent (1970) and Somasegaran *et al*. (1994). Inoculation of seeds by *Bradyrhizobium* sp. (Lotus) was carried out according to the method recommended by Silini *et al*. (2016).

The seeds were germinated in Petri dishes (20 seeds per dish) whose bottom was covered with a double layer of filter paper soaked in distilled water (control), solutions of different concentrations of $CdCl_2$ and $CuCl_22H_2O$ (Sigma-Alrdich; purity 99.99%) (tests). Germination was carried out in the dark at a temperature of 25 °C (Mihoub *et al*., 2005) (Table 1).

Table 1. Concentration of copper and cadmium used (µg ml^{-1}).

Concentration (µg ml^{-1})	Cu(II)	Cd(II)	Cu(II): Cd(II)	
C1	1 500	2 000	I	1 500:2 000
C2	2 000	3 500	II	2 000: 3 500
C3	3 000	6 000	III	3 000:6 000

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Cumulative germination rate

The cumulative germination rate (TCG) was determined according to the following formula (Bouton *et al*., 1976).

$$TCG\ \% = \left[\frac{G2}{2}\right] + \left[\frac{G4}{4}\right] + \left[\frac{G6}{6}\right]$$

Where: G2, G4 and G6 are the germination percentages at 2, 4 and 6 days after the initiation of germination.

Germination kinetics

The number of germinated seeds was recorded every 24 hours for seven days.

Mean daily germination

It is the ratio between the percentage of final germination (TG%) and the number of days to final germination (N) designated by MDG (Osborne *et al*., 1993).

$$MDG = \frac{TG\%}{N}$$

Germination speed

The germination speed (TMG) is calculated according to the following formula (Come, 1970),

$$TMG(day) = \frac{(N_1.T_1) + (N_2.T_2) + \dots\dots + (N_n.T_n)}{(N_1 + N_2 + \dots\dots + N_n)}$$

Where: N_1: is the number of seeds germinated at time T1; N_2: is the number of seeds germinated in the interval T1 -T2.

Inhibition of germination

The percentage inhibition (I%) of germination was calculated according to El Hadji-Djibo *et al*. (2014) as follows:

$$I\% = \left[\frac{Xi - Yi}{Xi}\right] \times 100$$

Where: Xi= number of seeds having germinated on the control medium; Yi= number of seeds having germinated on the medium containing Cu(II) or Cd(II).

Reversibility of the action of copper and cadmium

The 20 seeds germinated in presence of C1, C2 and C3 of trace metals (TM) for seven days were used. Among these seeds we chose those that were not germinated. They were rinsed three times then transferred to medium containing distilled water for twenty additional days. The percentage of germination recovery (RG%) was determined by the following formula (Bennani *et al*., 2015):

$$RG\% = \frac{(a-b)}{(c-b)} \times 100$$

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Where: a= total number of seeds germinated after transfer to distilled water; b= total number of seeds germinated on solution containing TM; c= total number of seeds germinated.

Toxicity of ions and osmotic effects on germination

Ten non-inoculated seeds were placed on a medium supplemented with 10 ml of solution containing different concentrations of cadmium and copper or iso-osmotic solutions of mannitol (Bennani *et al*., 2015) (Table 2).

Table 2. Concentration of iso-osmotic mannitol solutions (g L^{-1}).

		Cu(II)			Cd(II)			Cu(II)+Cd(II)	
TM (µg ml^{-1})	1 500	2 000	3 000	2 000	3 500	6 000	I	II	III
Mannitol (g L^{-1})	5.08	10.16	15.24	0.5	1.01	1.52	6	11.18	16.77

The incubation lasted 20 days (Bennani *et al*., 2015). Van't Hoff's law is used to calculate the iso-osmotic solutions of mannitol:

$$\pi = RT\left({}^{n}\!/_{V} \right) i\, \varphi$$

Where: π= osmotic pressure (Pa); R= ideal gas constant 8.314 (UI); T= absolute temperature in°K 273 + temperature in °C; n= number of moles of solute; V= volume (m^3); i= number of particles formed by dissociation of the solute; φ(phi)= osmotic coefficient= correction factor

Effect of the combination of copper and cadmium

The type of interaction between the two TM was evaluated by Abbott's formula (Gisi, 1996). In this model, the inhibition of theoretical germination of the mixture, I_{th}, expressed as a percentage, was determined according to the following formula:

$$I_{th}= I_A+I_B-\left(\frac{I_A \quad I_B}{100}\right)$$

Where I_A and I_B represent the inhibitions caused by the TM alone (copper and cadmium respectively). The inhibition ratio (RI) corresponding to each combination was calculated according to the following equation:

$$RI = \frac{I_{obs}}{I_{th}}$$

Values of (RI) greater than 1 indicate a synergy between the two TM; values of (RI) less than 1 mean antagonism between the two TM; values of (RI) equal to 1 correspond to an additivity of effects.

Statistical analysis of data

The statistical analysis of the results was performed using SPSS© software version 20.0.0 for Windows™. The experiments were replicated six times, consistently showing similar trends according to Shapiro-Wilk normality test (p= 0.89 > 0.05). Manova tests were conducted at a 5% significance level to evaluate the impact of bacterial inoculation on parameters. Additionally, the effect of *Bradyrhizobium* sp. (Lotus) on ion toxicity during germination in metallic conditions was assessed using the binomial test.

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Results

The results illustrate the effects of varying concentrations of copper and cadmium on the germination parameters of seeds, both with and without inoculation of *Bradyrhizobium* sp. (Lotus) (Table 3).

Table 3. Impact of *Bradyrhizobium* sp. (Lotus) inoculation on germination parameters.

TM	Conc	TCG (%)		MDG (seed days^{-1})		TMG (days)		Inhibition (%)	
		Nino	Ino	Nino	Ino	Nino	Ino	Nino	Ino
Control	0	86.89^{cB}	86.67 ^{iB}	33.01 ^{cB}	33.33 ^{fB}	2.01 ^{bB}	1.6 ^{aB}	0 ^{cA}	0 ^{aA}
Cu(II)	1 500	0 ^{aA}	10.83 ^{aB}	0 ^{aA}	2.86 ^{aB}	0 ^{aA}	3.5 ^{dB}	100 ^{aB}	80 ^{fA}
	2 000	0 ^{aA}	18.33 ^{fB}	0 ^{aA}	4.29 ^{bB}	0 ^{aA}	3.67 ^{eB}	100 ^{aB}	70 ^{eA}
	3 000	0 ^{aA}	17.5 ^{eB}	0 ^{aA}	4.29 ^{bB}	0 ^{aA}	2.5 ^{cB}	100 ^{aB}	70 ^{eA}
Cd(II)	2 000	0 ^{aA}	45 ^{hB}	0 ^{aA}	8.57 ^{eB}	0 ^{aA}	2.33 ^{bB}	100 ^{aB}	40 ^{bA}
	3 500	0 ^{aA}	16.67 ^{dB}	0 ^{aA}	7.14^{dB}	0 ^{aA}	4.1 ^{fB}	100 ^{aB}	50 ^{cA}
	6 000	0 ^{aA}	18.33 ^{fB}	0 ^{aA}	5.71 ^{cB}	0 ^{aA}	4.75 ^{iB}	100 ^{aB}	60 ^{dA}
Cu(II) + Cd(II)	I	4 ^{bA}	20^{gB}	1 ^{bA}	5.71 ^{cB}	4 ^{cA}	4.25^{gB}	90 ^{bB}	60 ^{dA}
	II	0 ^{aA}	15 ^{cB}	1 ^{bA}	4.29 ^{bB}	7 ^{dA}	3.67 ^{eB}	95 ^{bB}	70 ^{eA}
	III	0 ^{aA}	12.5 ^{bB}	0 ^{aA}	4.29 ^{bB}	0 ^{aA}	4.33 ^{hB}	100 ^{aB}	70 ^{eA}

Conc= TM concentration (µg ml $^{-1}$); Nino= on-inoculated seeds; Ino= inoculated seeds. Means followed by the same uppercase letter in the row, and lowercase in the column, do not differ statistically from each other according to Student's t-test and Tukey's test, respectively, at 5% of probability.

Effect of *Bradyrhizobium* sp. (Lotus) on the cumulative rate of germination

However, inoculation with *Bradyrhizobium* sp. (Lotus) led to an improvement in TCG values. In a cadmic environment, the presence of *Bradyrhizobium* sp. (Lotus) resulted in a TCG exceeding 45% at 2 000 µg ml^{-1} cadmium concentration, which decreased to 18.33% at 6 000 µg ml^{-1}. The germination of trefoil in a mixed copper and cadmium medium showed a proportional decrease in TCG with increasing concentrations. The highest TCG values were recorded at 1 500 and 2 000 µg ml^{-1} of Cu:Cd (20%). Overall, multivariate analysis of variance (Manova) confirmed that *Bradyrhizobium* sp. (Lotus) significantly impacted the cumulative germination rate (p= 0 < 0.05).

Effect of *Bradyrhizobium* sp. (Lotus) on germination kinetics

After seven days of metal treatment, the germination process can be divided into three distinct phases:

1) latency phase: in control conditions and with a 1 500 µg ml^{-1} cupric medium inoculated with *Bradyrhizobium* sp. (Lotus), this phase lasts approximately 24 h. However, in metal-stressed seeds with *Bradyrhizobium* sp. (Lotus) inoculation, this phase extends to 48 h. In contrast, without *Bradyrhizobium* sp. (Lotus) inoculation, particularly in seeds treated with metal mixtures (1 500:2 000 µg ml^{-1} and 2 000:3 500 µg ml^{-1} Cu:Cd), the latency phase lasts significantly longer, up to 96 and 144 h, respectively.

2) linear phase: seeds inoculated show a more pronounced initial increase in germination rate compared to non-inoculated seeds. However, this linear phase is absent in seeds subjected to different metallic treatments without *Bradyrhizobium* sp. (Lotus) inoculation.

3) final germination phase: It represents the final percentage of germination, reflecting the overall germination capacity under experimental conditions. In control conditions, the germination rate reaches its maximum (100%) after 3 to 4 days, regardless of *Bradyrhizobium* sp. (Lotus) inoculation.

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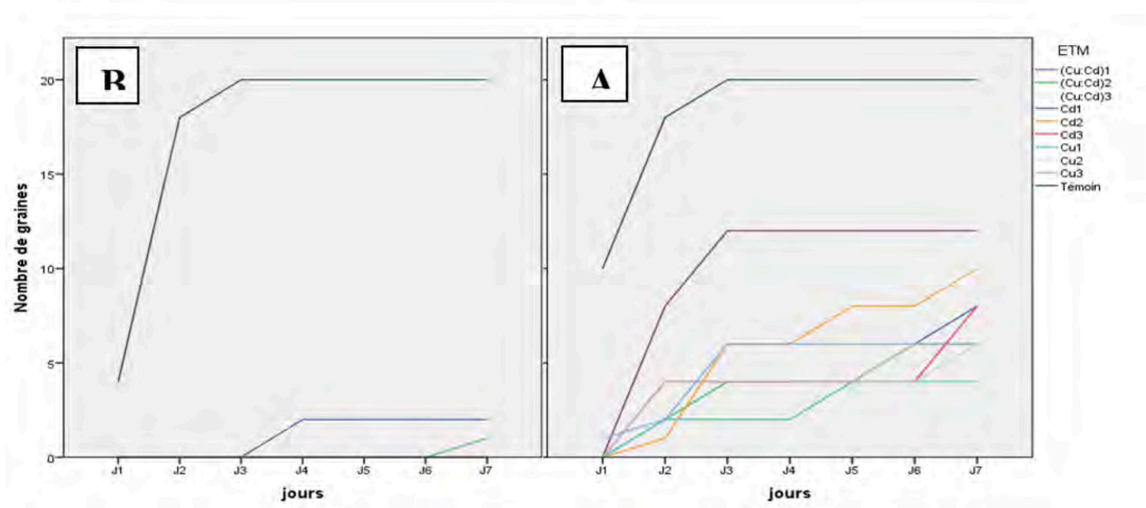
Under metal stress and with *Bradyrhizobium* sp. (Lotus) inoculation, the final germination rate is achieved much more rapidly compared to non-inoculated seeds under similar conditions (Figure 1).

Figure 1. Effects of metal stress on the germination kinetics of trefoil inoculated (A) and non-inoculated (B) with *Bradyrhizobium* sp. (Lotus)



Mean daily germination

Under metal stress without bacterial inoculation, the MDG drops to 0%, which is consistent across various concentrations of copper, cadmium, and combined copper-cadmium treatments (3 000:6 000 µg ml^{-1}).

The MDG remains notably low at concentrations of 1 500:2 000 µg ml^{-1} and 2 000:3 500 µg ml^{-1} in the combined copper-cadmium medium, at 1.43% and 0.71%, respectively. Inoculated seeds demonstrate higher MDG compared to non-inoculated seeds. With increasing cadmium concentrations, the MDG values decrease; for instance, at 2 000 µg ml^{-1}, the MDG reaches 8.75%, which is moderate compared to the control (33.33%).

The MDG declines proportionally with concentration increases. The lowest MDG is observed at a copper concentration of 1 500 µg ml^{-1} (2.86%), which increases to 4.29% at 2 000 and 3 000 µg ml^{-1}. Under metal stress in a cupric-cadmium medium with *Bradyrhizobium* sp. (Lotus) inoculation, the average MDG of trefoil seeds decreases with increasing metal concentrations.

The maximum value recorded is 5.71% for the 1 500:2 000 µg ml^{-1} (Cu:Cd) concentration. Multivariate analysis of variance (Manova) indicates a significant impact of *Bradyrhizobium* sp. (Lotus) on the average daily germination rate (p= 0< 0.05).

Germination speed

Seeds germinated in distilled water had a mean germination time (TMG) of 1.9 days. However, TMG could not be calculated for seeds germinated in pure cadmium or copper solutions due to the absence of germination across all concentrations tested. Under metal stress without bacterial inoculation, germination speed (TMG) decreased by 50% to 90% compared to the control when using a mixture of copper and cadmium, with reductions proportional to concentration except at 3 000:6 000 µg ml^{-1} (Cu:Cd) where no germination occurred.

The TMG of the control (1.6 days) decreased in the presence of *Bradyrhizobium* sp. (Lotus), indicating increased germination speed with bacterial inoculation. After inoculation, there was a reduction in germination speed proportional to cadmium concentration, reaching TMG= 4.75 days

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for 6 000 µg ml^{-1} of CdCl2. In a copper medium, TMG was 3.5 and 3.67 days for concentrations of 1 500 and 2 000 µg ml^{-1}, respectively.

Multivariate analysis of variance (Manova) revealed that *Bradyrhizobium* sp. (Lotus) significantly impacted germination speed (p= 0< 0.05).

Inhibition of germination

In the absence of *Bradyrhizobium* sp. (Lotus), the inhibition rate of germination was 100% when either copper or cadmium was applied separately, regardless of ion concentration, contrasting with the zero-rate observed in the control. The lowest inhibition rate was observed at concentration C1 of the copper-cadmium mixture (90%), with a 5% increase proportionate to concentration. The rate peaked at 80% for the C1 copper concentration and decreased to 70% with increased concentration.

For cadmium, the inhibition rate was lowest at 40% for concentration C1, increasing to 60% at C3. The copper-cadmium mixture showed an inhibition rate of 60% at C1, increasing to 70% comparable to copper alone. Despite these variations, the inhibition rates were notably lower than those in tests not inoculated with *Bradyrhizobium* sp. (Lotus). Multivariate analysis of variance (Manova) demonstrated that *Bradyrhizobium* sp. (Lotus) significantly influenced the germination inhibition rate (p= 0< 0.05).

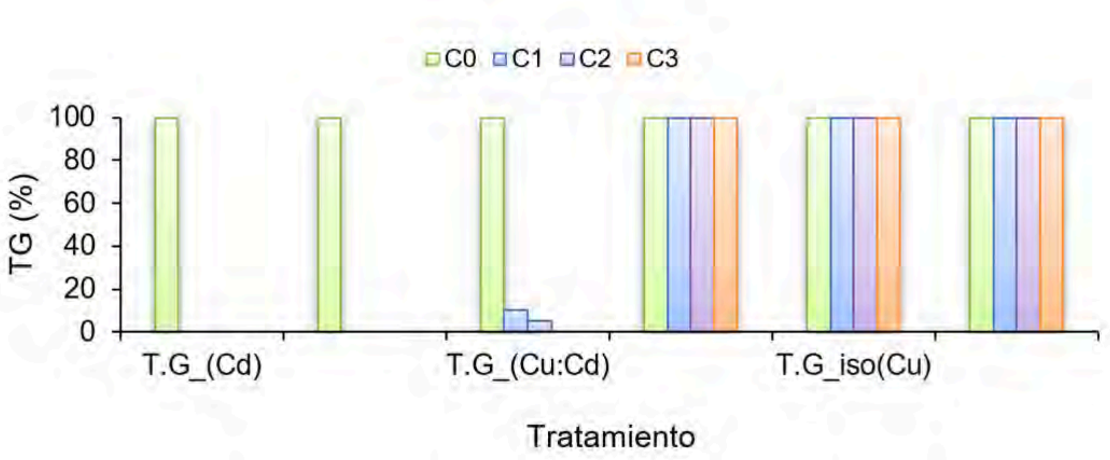
Reversibility of germination inhibition

It has been demonstrated in previous results that copper and cadmium and their combination exert at different concentrations, a depressive effect on seed germination. This inhibition can be osmotic and toxic. If it is of osmotic origin, we should expect a resumption of germination after lifting this constraint. However, if ionic toxicity phenomena occur, we can expect a lack of resumption of germination. We particularly noted an absence of resumption of germination for the trefoil regardless of the metallic and bacterial pretreatment. This confirms that the action of TM is toxic in nature.

Determination of ion toxicity on germination

To better prove the osmotic or toxic effect of TM on trefoil, we compared the germination behavior on metallic medium and on mannitol. The Trefoil v Mannitol interaction was significant (binomial= 0.002< 0.05). The germination percentages on mannitol (100%) were higher than those recorded at the different concentrations of TM (C1, C2 and C3) and this despite the osmotic pressure which indicates the toxic effects of the metallic ions (Figure 2).

Figure 2. Final germination rate of trefoil on iso-osmotic solutions (TG-iso) compared to different concentrations of TM.



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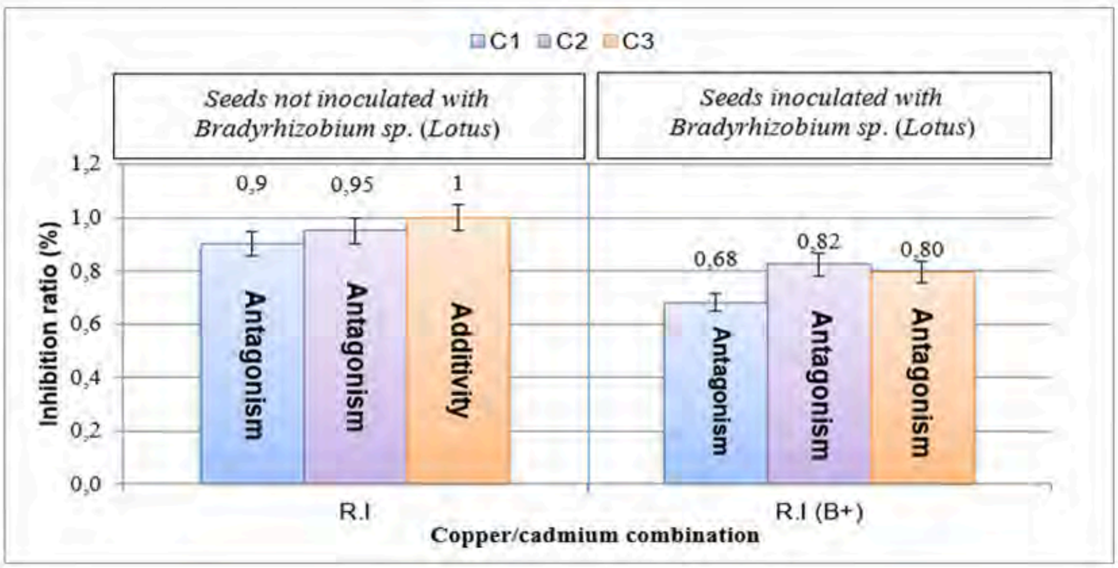
Combination of copper and cadmium

Without *Bradyrhizobium* sp. (Lotus), the germination inhibition ratio (RI) ranged from 0.9 to 1, showing a 5% increase proportional to concentration, suggesting antagonistic interactions between copper and cadmium, the RI was 1, indicating an additive interaction between the elements.

Inoculation with *Bradyrhizobium* sp. (Lotus) reduced the inhibition ratio by 13 to 22% compared to controls without bacterial inoculation. This reduction in RI with *Bradyrhizobium* sp. (Lotus) indicates an antagonistic interaction for all combinations. Notably, there was a shift from additivity to antagonism between copper and cadmium at the C3 concentration. Multivariate analysis of variance (Manova) showed that pre-inoculation of trefoil seeds with *Bradyrhizobium* sp. (Lotus) led to significantly different responses compared to non-inoculated tests (p= 0.02 < 0.05) (Figure 3).

Figure 3. Effect of *Bradyrhizobium* sp. (Lotus) on the germination inhibition ratio (RI) (%) and the copper/cadmium combination.



Discussion

Bradyrhizobium sp. (Lotus) demonstrates notable resistance to both copper and cadmium. In silico estimations suggest that the minimum inhibitory concentration (MIC) for cadmium is approximately 10 000 µg ml^{-1}, whereas for copper, it is 2 000 µg ml^{-1} (Degaïchia *et al*., 2024). This high tolerance of *Bradyrhizobium* sp. (Lotus) to these heavy metals positions it as a promising solution to tackle environmental pollution caused by trace metals.

The cumulative rate and the final germination percentage of trefoil seeds decrease with the increase in the concentration of TM in the medium. Indeed, our results highlight that excess copper and/or cadmium in the environment causes irreversible toxicity. It is the same for the speed, the average as well as the kinetics of germination. These results are aligned with those reported by Mihoub *et al*. (2005); Lamhamdi *et al*. (2011).

According to these authors, TM (copper, cadmium and lead, etc.) in the medium significantly inhibit the germination of seeds of some *Fabaceae*. Inoculation of trefoil seeds with *Bradyrhizobium* sp. (Lotus) significantly improves germination under metal stress.

According to Nelson (2004), *Rhizobia* can exert a beneficial effect on plant growth by increasing the cumulative rate of germination and their speed. The positive effect of *Rhizobia* on seed germination in unfavorable environments and the emergence of the coleoptile would be attributed to the bacterial

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capacity to produce or modify plant hormones including gibberellins which play a key role in germination (Barassi *et al*., 2006).

By analyzing the impact of TM on a multitude of vital physiological functions of the plant, Ernst (1998) admits that germination is a process that is certainly vulnerable to metal stress, but which would be one of the most resistant among the other phases of plant development.

The integumentary barriers of seeds would prevent a strong accumulation of metallic trace elements. Furthermore, for any physiological or metabolic process, it is the critical phytotoxicity thresholds, defined in terms of tissue accumulation, which determine sensitivity to TM (Woolhouse, 1983; Fernandes *et al*., 1991).

The inhibition of germination is extremely pronounced in the non-inoculated trials. This is due, as explained above, to the toxic effect of TM. *Bradyrhizobium* sp. (Lotus) induces a significant reduction in this inhibition.

Conclusions

The results demonstrate that *Bradyrhizobium* sp. (Lotus) inoculation significantly improves germination parameters under metal stress conditions. Inoculation increased the total germination percentage (TCG) and reduced the germination latency induced by trace metal stress. Seeds treated with *Bradyrhizobium* sp. (Lotus) exhibited higher TCG values and shorter median germination times (TMG) compared to untreated seeds, particularly at higher copper and cadmium concentrations.

The use of *Bradyrhizobium* sp. (Lotus) represents a promising strategy to enhance trefoil germination under trace metal stress, indicating the potential of this symbiotic bacterium to enhance plant tolerance to trace metal elements during germination. These findings provide valuable insights for the development of sustainable agricultural practices aimed at mitigating the adverse effects of metal stress on crop productivity.

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