
Effects of high-dose 2,4-D on germination and initial development of *Canavalia ensiformis* (L.) DC.

Efeitos de doses altas de 2,4-D na germinação e desenvolvimento inicial de *Canavalia ensiformis* (L.) DC.

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RESUMO

Este trabalho teve como objetivo avaliar a germinação e desenvolvimento inicial de *Canavalia ensiformis* sob altas concentrações de ácido 2,4-diclorofenoxiacético (2,4-D), em diferentes substratos. Na primeira análise, sementes de diferentes procedências foram descontaminadas e introduzidas em um vaso Leonard modificado (vaso PET) contendo substratos de areia lavada ou areia lavada mais vermiculita, sob altas concentrações de 2,4-D (0 - controle, 250 e 500 mg/L). Para uma segunda análise, em substrato areia lavada esterilizada, as sementes foram observadas quanto a germinação, calogênese e contaminação em diferentes concentrações de 2,4-D (0 - controle, 20, 40, 250 e 500 mg/L). Uma terceira análise foi realizada com areia lavada estéril e areia de mata ciliar estéril e não estéril com 2,4-D (40 mg/L) e controles. Os resultados mostraram que maiores concentrações de 2,4-D induziram maiores áreas de formação de calos nas sementes. Na concentração de 40 mg/L de 2,4-D, a germinação em substrato de mata ciliar mais areia lavada resultou no crescimento de raízes e formação de calos, sem contaminação. As sementes de *C. ensiformis*, apesar de serem tolerantes a altas concentrações de 2,4-D, após germinação, as plântulas apresentaram danos no desenvolvimento inicial.

Palavras-chave: Fabaceae; Adubo verde; Feijão de porco; Herbicida

ABSTRACT

This study aimed to evaluate seed germination and initial development of *Canavalia ensiformis* under high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) in different substrates. In the first analysis, seeds from different origins were disinfected and introduced into a modified Leonard vessel (PET vessel) containing washed sand or washed sand plus vermiculite substrates and under high concentrations of 2,4-D (0 - control, 250 and 500 mg/L). For a second analysis, seed germination, callogenesis and contamination in different concentrations of 2,4-D (0 - control, 20, 40, 250 and 500 mg/L) were evaluated with sterile washed sand as substrate. The third analysis was carried out with sterile washed sand and sterile and non-sterile riparian forest sand with 2,4-D (40 mg/L) and control. Results showed that higher concentrations of 2,4-D induced larger areas of callus formation. At a concentration of 40 mg/L of 2,4-D, germination in riparian forest substrates washed sand resulted in root growth and callus formation without contamination. Although *C. ensiformis* seeds showed tolerance to high concentrations of 2,4-D, after germination, the plantlets presented damage in the initial development.

Keywords: Fabaceae; Green manure; Jack bean; Herbicide

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INTRODUCTION

Canavalia ensiformis (L.) DC. (Fabaceae, Leguminosae), or jack bean, is widely used as a green manure because it presents rapid growth and adaptation to diverse edaphoclimatic conditions (AMBROSANO *et al.*, 2018), as well as N₂ fixation in symbiotic association with diazotrophic bacteria. As such, its use contributes to improving the chemical, physical and biological features of soils (MARTÍN *et al.*, 2007). In addition, studies of phytoremediation have shown that mycorrhizal colonization of roots of this species is an important resource for its survival in contaminated soils and removal of pollutants (ANDRADE *et al.*, 2010). Green manure brings numerous benefits that promote the maintenance and conservation of agro-systems (TEODORO *et al.*, 2014).

For agriculture, 2,4-D (2,4-dichlorophenoxyacetic acid) is mainly applied to eliminate weeds in monocot crops, such as wheat and sugarcane. It can also be used in eudicotyledonous crops, such as coffee and soybeans, without direct contact in the cultures. In *in vitro* culture, it is used to mimic the effect of auxins on plant physiology. However, regardless of the recommended use of 2,4-D, it is a chemical substance associated with toxicity and physiological changes in ecosystems (VIGÁRIO and MORAES, 2014; FRIEDRICH, 2014; COSTA *et al.*, 2020). Its use in high concentrations can cause effects over long distances owing to leaching through water flow, mainly pluviometric. Retention in the environment's water and soil depends on its features (SILVA *et al.*, 2010; DIAS *et al.*, 2011; GARCÍA *et al.*, 2022). Among its many harmful effects, the inhalation of 2,4-D herbicide can cause gastrointestinal and neuromusculature problems in addition to its association with soft tissue sarcomas and endocrine disruptor compounds (EDC) (BRADBERRY *et al.*, 2004; VIEIRA *et al.*, 2021). In plants, 2,4-D is absorbed by roots, stems and leaves, so the herbicide is translocated to the meristems of eudicotyledonous plants (MUNRO *et al.*, 1992). According to the United States Environmental Protection Agency, 2,4-D harms and kills plants in three ways. It alters the plasticity of cell walls and increases ethylene associated with senescence. The amount of protein it produces can also be harmful. However, combining the planting of green manure with phytoremediation benefits agriculture since the plant helps to nourish the soil in relation to nitrogen and reduces contamination by 2,4-D used as a herbicide (SILVA *et al.*, 2019).

Therefore, plants used for phytoremediation must tolerate high concentrations of 2,4-D. *C. ensiformis* is a legume that has shown high mean values for the fresh and dry mass production that is relevant for use as green manure (TEODORO *et al.*, 2014). Previous research on the use of green manures for phytoremediation of herbicides has shown the potential of *C. ensiformis* and

Crotalaria juncea L. in the removal of herbicides, such as sulfentrazone, from the soil (OLIVEIRA *et al.*, 2014; BELO *et al.*, 2016; FERRAÇO *et al.*, 2017), as well as the potential of *C. ensiformis* (SILVA *et al.*, 2019) and *C. juncea* (COSTA *et al.*, 2022) in the removal of 2,4-D herbicide.

However, whether green manures can withstand high concentrations of 2,4-D and still manage to develop remains to be elucidated. Essentially, green manures serve a dual purpose: fertilization and remediation of 2,4-D from areas that suffer contamination by herbicides. Thus, the present work aimed to evaluate the tolerance of *C. ensiformis* plants to high concentrations of 2,4-D in a developmental context. For cultivation of *C. ensiformis*, commercial washed sand was used, as well as riparian forest substrate, considering plant development in different types of substrates. For this purpose, the riparian forest of Guandu River was chosen to collect substrate in order to evaluate *C. ensiformis* development with an eye toward contributing toward the recovery of riverine ecosystem through *in situ* sowing of seeds.

METHODOLOGY

Experimental area

The experiment was carried out between November 2017 and February 2018 on the campus of the Universidade do Estado do Rio de Janeiro (UERJ-ZO) in the West Zone, RJ (22°53'58.1"S, 43°34'45.1"W) and under natural light and temperature conditions (Table 1). Vessels were protected by a transparent plastic tarp on the roof and sides in a shaded location under environmental conditions.

Table 1 - Environmental conditions of the experiment

Temperature (°C)		Air Humidity (%)	
	Nov-Dec	Jan-Feb	
Min.	20 ± 2	20 ± 2	80
Max.	30 ± 2	28 ± 2	90

Source: CPTEC/INPE

Seeds and Substrates

The seeds used were obtained from Futuro Fértil (FF) (S. T. Irajá Agrícola Ltda.) located in the State Supply Centers (Ceasa - RJ). These seeds belonged to the 2015 harvest, lot 08/2015, presenting purity of 98% and germination rate of 75%. Seeds donated from Pirai Sementes (PS) were also used to evaluate germination. These seeds belonged to the 2017 harvest, presenting purity of 98% and germination rate of 75%.

Two substrates were used: washed sand plus vermiculite and roadside substrate from a riparian forest of Guandu River with depth up to 10 cm (Estrada Dique do Guandu) in soil bordering the river in Rio de Janeiro State (22°45'59.6"S 43°38'14.07"W).

Asepsis of seeds and preparation of vessels

For disinfestation, seeds were soaked in 70% alcohol for 2 min, followed by commercial sodium hypochlorite from 2% to 2.5% for 3 min. Seeds were washed with distilled and sterile water 3 times between steps. Seed manipulation was done in a laminar flow. The number of seeds used varied according to the test.

The seeds were introduced into modified Leonard's vessels (VINCENT, 1970), i.e., polyethylene terephthalate vessels (SILVA *et al.*, 2019), termed as PET vessels, containing substrate and nutrient solution. The substrates used were sterilized by autoclave: washed sand, washed sand plus vermiculite (1:1), and riparian forest substrate (sterile and non-sterile). For nutritive solution, a 1000 L Hidrogood® fert nutrient kit was used (Hidrogood Horticultura Moderna Ltda., Taboão da Serra - SP). The nutrient solution was prepared using 16 g/L of water which was poured into the lower part of the PET vessel and then transferred to the substrate through a string by capillarity (SILVA *et al.*, 2019). The Hidrogood® solution contents consisted of 10% nitrogen, 9% phosphorus pentoxide, 28% potassium oxide, 3.38% magnesium, 4% sulfur, 0.06% boron, 0.01% copper, 0.05% manganese, 0.0729% molybdenum and 0.02% zinc.

The amounts added to each vessel were 300 g of substrate and 300mL of nutrient solution. The substrates were sterilized for one hour at 121°C for two consecutive days in an autoclave. After cooling, the sterile substrate was placed in the upper part of the vessel. The nutrient solution containing 2,4-D was also sterilized using an autoclave.

Herbicide 2,4-dichlorophenoxyacetic acid

The concentrations of 2,4-D used in this work corresponded, respectively, to the application of Dow AgroSciences DMA 806BR herbicide with active ingredient 2,4-D in the concentration

of 0.2, 1.4 and 2.8 L/ha. According to the herbicide leaflet, dosage depends on the crop for which the herbicide is intended, ranging from 0.3 to 3.5 L/ha, but for most crops, the recommended application values range from 1 to 2 L/ha of the commercial product (Dow AgroSciences, 2018). The high concentrations of 2,4-D used varied from 0 (control) to 20, 40, 250 and 500 mg/L.

Seed germination and seedlings

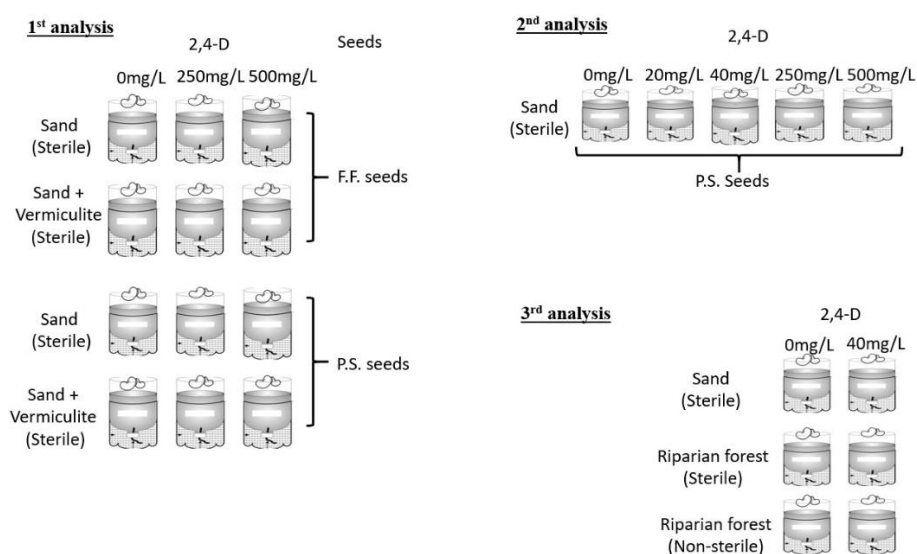
In the first analysis, washed sand and washed sand plus vermiculite were used, both sterile. Futuro Fértil (FF) and Piráí Sementes (PS) seeds were tested. The seeds were placed in concentrations without 2,4-D (control) and concentrations of 250 and 500 mg/L of 2,4-D. For each concentration of 2,4-D, 2 seeds were used in each vessel, totaling five PET vessels x 2,4-D concentration, $n = 10$ seeds. At this stage, the results of seed germination of different origins and with two substrates were compared, making it possible to proceed to the second stage using seeds with the most suitable substrate.

The second analysis used sterile washed sand as substrate and only seeds from PS. The 2,4-D concentrations applied were 0 (control), 20, 40, 250 and 500 mg/L of 2,4-D. The n for each concentration was 60 seeds.

The third analysis was carried out with sterile washed sand and sterile and non-sterile riparian forest sand. The 2,4-D concentration used was 40 mg/L and control; five PET vessels were used with two seeds in each.

All analyses are summarized in the scheme below (Figure 1).

Figure 1 – Scheme of experimental analysis



Evaluation of Parameters

Germination was established from root protrusion. Germination and seedling development were observed occasionally at 4, 10, 13 and 30 days. Germination percentage and velocity index (GVI) were evaluated in all tests.

Germination velocity index (GVI) was calculated as:

$$GVI = \frac{G_1}{N_1} + \frac{G_2}{N_2} + \dots + \frac{G_n}{N_n},$$

Where, GVI = Germination Velocity Index, G = number of germinated seeds, and N = number of days after sowing in which counting was carried out.

Only in the second evaluation were the following seedling development parameters observed: length of hypocotyl and epicotyl (length of shoot), number of leaves, length of roots and contamination at 4, 7, 10, 13 and 30 days.

Statistical Analysis

Analysis of variance of the data obtained was performed, and in the comparison of means, the Tukey test was used at 5% probability, using BioEstat 5.0.

RESULTS AND DISCUSSION

Germination of *Canavalia ensiformis* seeds

In the first analysis, control showed a germination percentage of 20% and 40% for FF seeds and PS seeds (sand plus vermiculite), respectively (Tables 2 and 3). The use of vermiculite did not change the percentage of seed germination from PS, which was also 40% compared to control (without vermiculite) (Tables 2 and 3). The seeds from FF had a low germination percentage (10 and 20%) in the control treatment or without vermiculite. On average, *C. ensiformis* seeds needed 4 days for first emergence.

Table 2 – Total percentage of germination of *Canavalia ensiformis* seeds after 4 days of sowing, using vermiculite substrate plus washed sand, n= 10.

2,4-D(mg/L)	Germination (%)			
	FF	GVI	PS	GVI*
Control	20	0.5	40	1.0
250	0	0	10	0.25
500	0	0	0	0

*GVI= Germination Velocity Index. FF – Sementes da empresa Futuro Fértil. PS – Sementes da empresa Piráí sementes. No significant difference was shown by Tukey, $p < 0.05$.

The addition of 2,4-D impaired seed germination rate. With the addition of 250 mg/L of 2,4-D, a 50% reduction in the germination of seeds was observed from PS (Table 2), and at the concentration of 500 mg/L of 2,4-D, germination of these seeds was not verified independently according to the presence or absence of vermiculite (Tables 2 and 3). No significant difference was noted between seeds from FF and PS in either washed sand or washed sand plus vermiculite substrates. However, the seeds from the PS in the washed sand substrate were chosen for the second analysis since results of GVI showed better results.

Table 3 - Total percentage of germination of *Canavalia ensiformis* seeds after 4 days of sowing, using washed sand substrate, n= 10.

2,4-D (mg/L)	Germination (%)			
	FF	GVI	PS	GVI*
Control	10	0.25	40	1.0
250	0	0	20	0.5
500	10	0.25	0	0

*GVI= Germination Velocity Index. FF – Sementes da empresa Futuro Fértil. PS – Sementes da empresa Piráí sementes. No significant difference according to Tukey, $p < 0.05$.

Younger seeds of *C. ensiformes* (PS - 2017 harvest, purity = 98%) had higher germination percentages in controls (40%) and higher GVI (1.0), compared to older tested seeds (FF - 2015 harvest, batch 08/2015, purity = 98%), which had low germination (20%) and lower GVI (0.5). However, the substrate containing washed sand plus vermiculite was not as efficient as washed sand. The use of washed sand is advantageous owing to its low cost, physical stability and chemical inactivity, in addition to the ease of cleaning and disinfection (Sodré *et al.*, 2007). The sterilization process also ensures less interference from microorganisms.

Considering the results of the first analysis (Tables 2 and 3), an evaluation was proposed with PS for using sterile washed sand with different concentrations of 2,4-D. For each treatment, 30 PET vessels with 2 seeds each were used, $n = 60$ seeds per concentration. Regardless of the 2,4-D concentration used, a reduction in the percentage of seed germination was observed at the highest concentrations of 2,4-D (40, 250 and 500 mg/L) (Table 4). The control obtained the best germination rate (51.6%) (Table 4). Root protrusions were observed from the 4th day onwards.

GVI results were high since the concentration of 20 mg/L presented an index of 7.5, which was similar to that of control. Concentrations of 250 and 500 mg/L of 2,4-D showed the lowest GVI values (5.0).

Table 4 - Germination of *Canavalia ensiformis* on the 4th day after sowing in washed sand substrate containing different concentrations of 2,4-D.

2,4-D (mg/L)	Germination (%)	GVI*
Control	51.6 ^a	7.7
20	50.0 ^b	7.5
40	43.3 ^c	6.5
250	33.3 ^d	5.0
500	33.3 ^d	5.0

*GVI= Germination Velocity Index. Significant difference was noted between the averages; values accompanied by the same letter do not differ by the Tukey test, $n = 60$, $p < 0.05$.

After germination, the seeds showed good development in the control treatment, according to such parameters as hypocotyl and epicotyl length, root length and number of leaves (Figures 2 and 3). In the control, development averages were 5 cm for hypocotyl, 6 cm for epicotyl and 1 leaf up to the 13th day. The root showed an average of 2.8 cm up to the 30th day after sowing in the control condition (Figures 3C). On the 13th day, the control seedlings showed normal leaf and root development (Figure 2D). It was possible to observe how radicle length increased up to the 30th day after sowing, but only in the control treatment because in the other concentrations, 2,4-D was toxic, preventing the development of seedlings (Figure 3C).

Figure 2 - Illustration of germination and initial development of *Canavalia ensiformis* from controls in washed sand substrate (without 2,4-D). A) 4th day after sowing. B) 7th day after sowing. C) 10th day after sowing. D) 13th day after sowing.

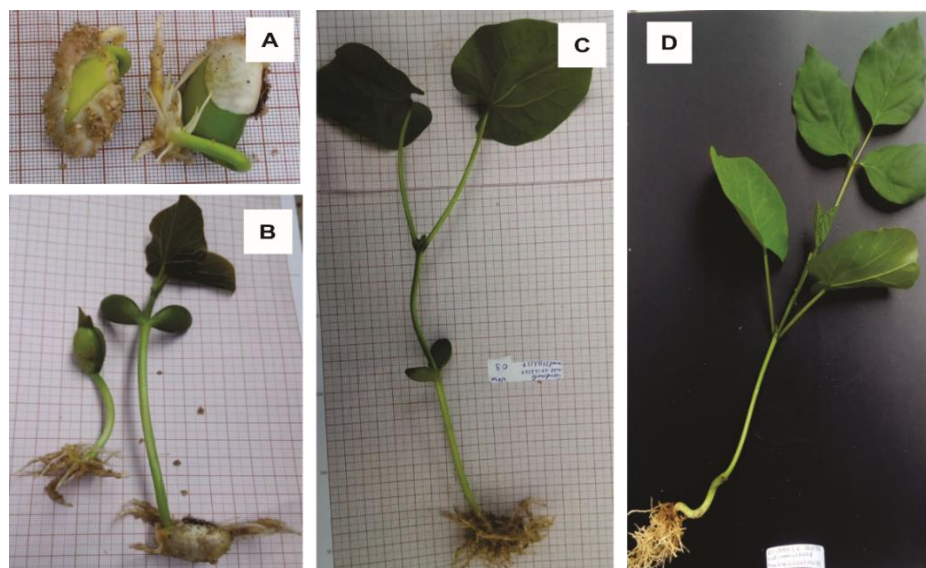
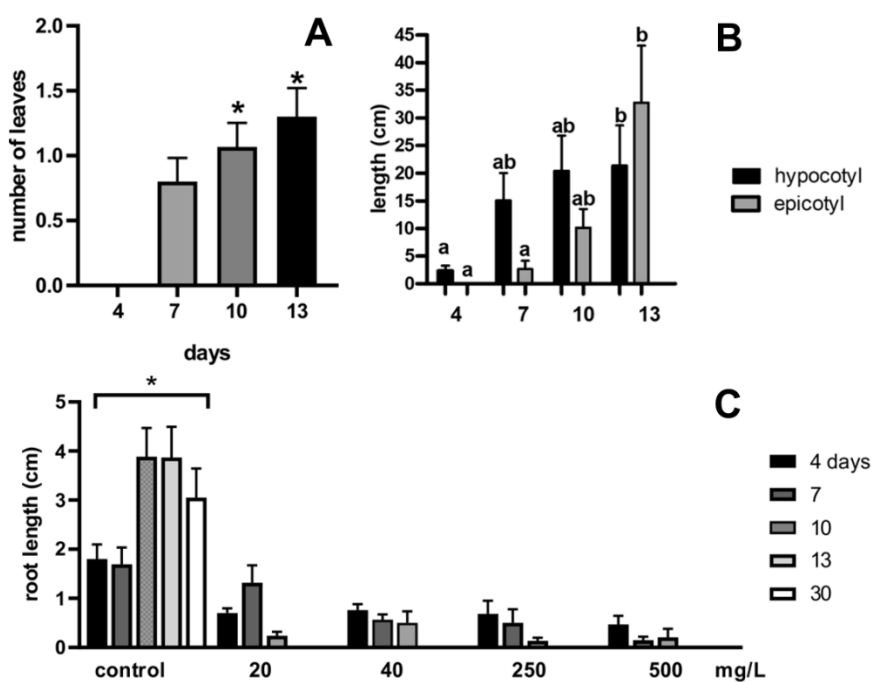


Figure 3 - Parameters for assessing the initial development of *Canavalia ensiformis* grown in PET vessels, washed sand substrate, over 13 days after germination: A) Radicle length after germination in different concentrations of 2,4-D. At 13 and 30 days, it was not possible to obtain length data owing to the death of the plants submitted to the 2,4-D concentrations. B-C) Length of hypocotyl and epicotyl (control). C-D) Developing leaves (number of leaves) and root length (control). Values are expressed as the mean. Error bars indicate standard error, n= 60. Different letters and (*) indicate statistical differences, Tukey, $p < 0.05$.

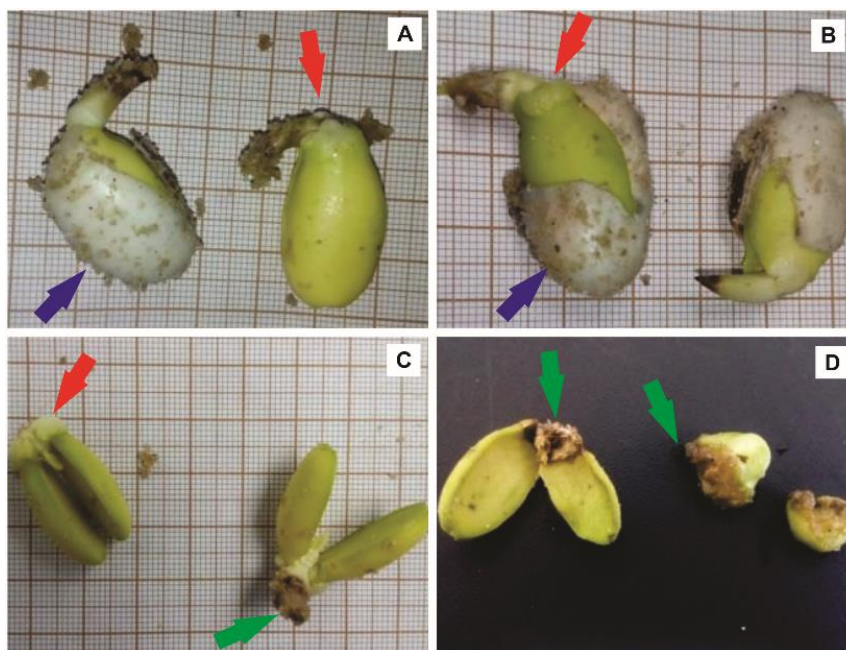


In treatments with 2,4-D, radicle growth was impaired (Figure 3C). Reduced radicle growth was observed until the 7th day at concentrations with 20 and 40 mg/L of 2,4-D, which

reached around 0.5 cm in contrast to the radicles of seedlings maintained in the control treatment that grew an average of 1.25 cm in the same period. Within 10 days, these seedlings had already stagnated in their development since the aerial part could not be seen (hypocotyl, epicotyl and leaves). Concentrations of 250 and 500 mg/L of 2,4-D resulted in damage to the physiological processes of germination as some seeds did not germinate and were eliminated, or, after germination, an inhibition of radicle growth was observed according to data on length of smaller radicle at 10 days compared to that at 7 days and no growth at 13 and 30 days (Figure 3C). At the end of the experiment, all plants subjected to concentrations of 20 to 500 mg/L of 2,4-D had signs of damage to their physiological responses along development (Figure 3C).

Figure 4 shows undesirable callus formation from the 4th day with development up to the 10th day after cultivation. Calluses have a wavy appearance and whitish color (red arrows). On the 30th day, contaminated areas were observed in callused areas with a brownish appearance owing to oxidation, which initiates the decomposition of plant matter (green arrows).

Figure 4 - Germination, callus formation and contamination of *Canavalia ensiformis* seeds from the 20 mg/L 2,4-D treatment. A) 4th day after sowing, when germination was observed. B) 7th day after cultivation (callus formation). C) 10th day after sowing (presence of callus affecting development). D) 13th day after sowing (contamination). Blue arrows indicate seed coat, red arrows indicate calluses, and green arrows indicate contamination.

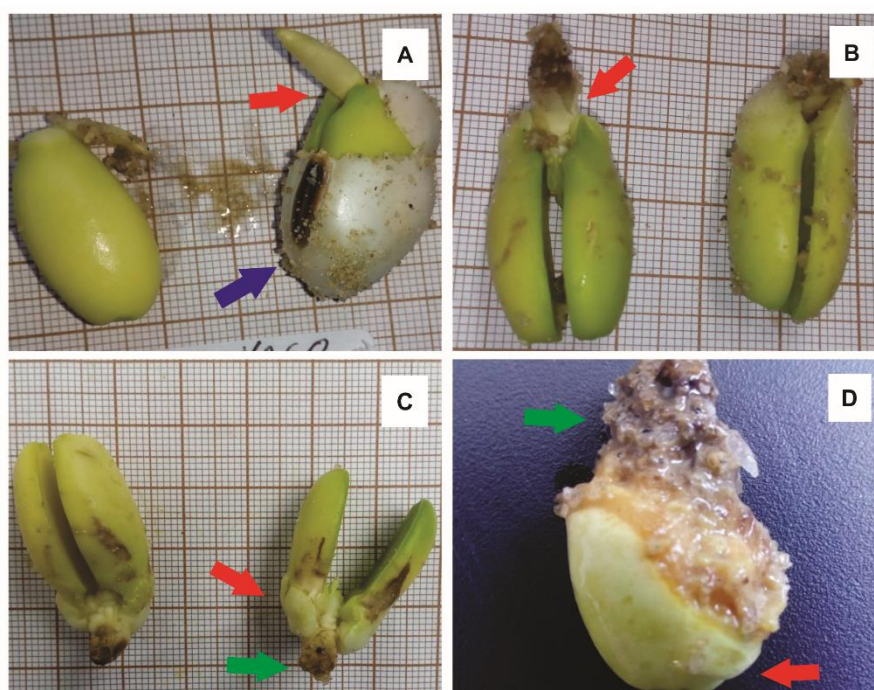


As shown in Figure 3C, the length of radicles had declined by the 10th day after sowing the seeds, as better visualized in Figure 5, showing that the seed on the 10th day with part of the radicle already contaminated (Figure 4C). In Figure 4D, the cotyledon is contaminated, mainly in

the radicle region, and owing to this contamination, 2 cotyledons of the seed had loosened. Callus formation was also observed on the cotyledon and radicle of germinated seedlings (Figure 4A to D).

In Figure 5, the radicle also presents contamination and reduction in mean length, as can be seen in Figure 3C for seeds exposed to a concentration of 40 mg/L of 2,4-D.

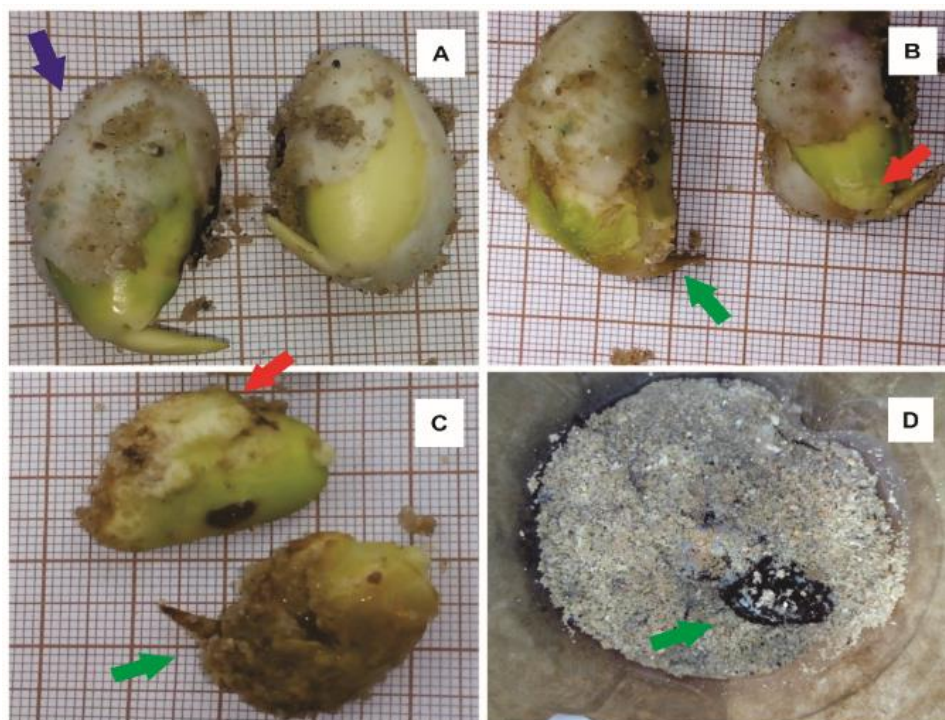
Figure 5 - Germination, callus formation and contamination of *Canavalia ensiformis* seeds from 40 mg/L 2,4-D treatment. A) 4th day after sowing (germination). B) 7th day after sowing (callogenesis). C) 10th day after sowing (presence of callus affecting development). D) 13th day after sowing (contamination). Blue arrows indicate seed coat, red arrows indicate calluses, and green arrows indicate contamination.



In Figure 6A and 6B, the seeds still have their seed coats. Soon after radicle emission, brownish regions were observed in the hypocotyl-radicular axis that indicate oxidation (Figure 6B and 6C) and the presence of microorganisms (Figure 6D). Callus formation was observed on the cotyledon, appearing as whitish elevations (Figure 6). In Figure 6, contamination of the areas close to the radicle (Figure 6B-C) and the seed was observed. This seed was introduced into the sterile sand substrate in a PET vessel.

Figure 6 - Germination, callus formation and contamination of *Canavalia ensiformis* seeds at 250 mg/L 2,4-D treatment. A) 4th day after sowing (germination). B) 7th day after sowing (callus formation). C) 10th day after sowing (presence of callus affecting development). D) 13th day

after sowing (contamination). Blue arrows indicate the developing seed coat, red arrows indicate calluses, and green arrows indicate contamination.



In Figure 7B, we can see the contamination under the seed surface on the 7th day, along with the presence of callus and tegument, while on the 10th day of cultivation, the contamination increased, interfering with the development of the radicle (Figure 7C). Thirteen days after sowing, we can see an oxidized, dark and necrotic appearance (Figure 7D).

Calli formation on seed cotyledon was observed from the 4th day after sowing, except in the control treatments, following which the presence of callus was not observed (Table 5).

None of the control treatments showed contamination after seed germination (Table 6). In contrast, seeds submitted to treatments with 2,4-D showed a progressive contamination rate up to the 10th day of analysis after sowing (Table 6). On the 13th day after sowing, all seeds showed tissue damage and surface contamination (Table 6).

Figure 7 - Germination, callus formation and contamination of *Canavalia ensiformis* seeds from treatment with 500 mg/L of 2,4-D. A) 4th day after sowing (germination). B) 7th day after sowing (callus formation). C) 10th day after sowing (presence of callus affecting development). D) 13th day after sowing (contamination). Blue arrows indicate seed coat, red arrows indicate calluses, and green arrows indicate contamination

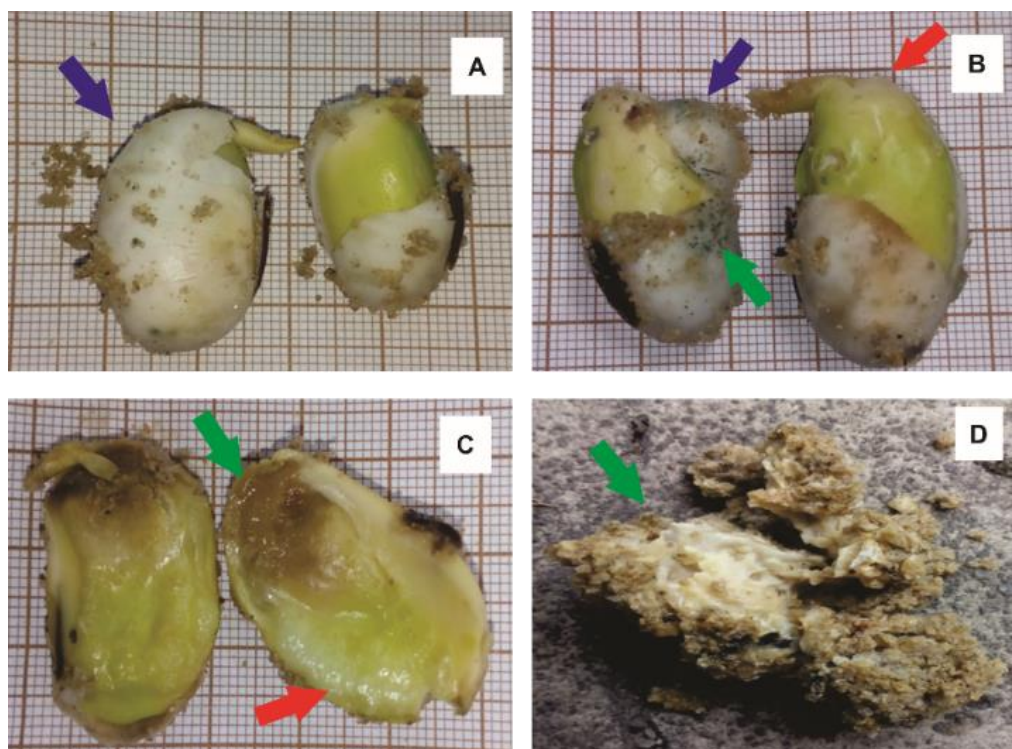


Table 5 - Callogenesis rate from the 4th day of sowing of *Canavalia ensiformis* under different concentrations of 2,4-D.

2,4-D (mg/L)	Callogenesis (%)		
	4th	7th	10th
Control	0.0 ^a	0.0 ^a	0.0 ^a
20	23.3 ^a	43.3 ^{ab}	66.6 ^b
40	3.9 ^a	65.3 ^b	80.7 ^b
250	5.0 ^a	85.0 ^b	90.0 ^b
500	0.0 ^a	65.0 ^b	70.0 ^b

Different letters indicate significant difference by the Tukey test, n= 60, p< 0.05.

Table 6- Contamination rate of *Canavalia ensiformis* seeds analyzed on days 4, 7, and 10 after cultivation in all concentrations of 2,4-D.

2,4-D (mg/L)	Contamination (%)		
	day 4	day 7	day 10
Control	0.0 ^a	0.0 ^a	0.0 ^a
20	6.7 ^a	50.0 ^b	50.0 ^b
40	3.8 ^a	25.0 ^{ab}	76.5 ^b
250	5.0 ^a	18.8 ^{ab}	69.2 ^b
500	25.0 ^a	44.4 ^{ab}	60.0 ^b

Different letters indicate significant difference by the Tukey test, n= 60, p< 0.05 among treatments.

Canavalia ensiformis showed germination capacity at all 2,4-D concentrations, control, 20, 40, 250, and 500 mg/L, grown in a PET vessel. A reduction in germination percentage and GVI values was observed at higher concentrations of 2,4-D. Seedling development was not observed at any concentration. At all concentrations, emission of radicle was noted. 2,4-D promotes an auxinic effect on plant development at low concentrations (XU *et al.*, 2015). Among responses associated with the physiological action of 2,4-D, we can mention callogenesis. The presence of calli in damaged plant tissue is widely used in plant tissue culture studies, such as the induction of somatic embryogenesis (GOMES-COPELAND *et al.*, 2017; CIPRIANO *et al.*, 2018; COSTA *et al.*, 2020). Auxinic action refers to the stimulation of cell division, as verified in the responses observed in seeds and radicles of cell proliferation forming the callus.

According to Grossmann (2009), the callus seems to become necrotic, as observed in this work, and consequently culminates in the death of the seedling. According to the literature, 2,4-D increases the production of ethylene, which can trigger a metabolic reaction that increases ROS (reactive oxygen species) capable of altering the production of the hormone abscisic acid (ABA). ABA is related to the physiology of plant development: growth inhibition, seed dormancy and stomata closure (SONG, 2013; BHARATH *et al.*, 2021). This could explain why 2,4-D apparently acted as an inhibitor of the initial development of *C. ensiformis* after germination. Just as 2,4-D induced callus formation, it may also have damaged the cell wall causing contamination of the seeds by the growth of microorganisms since this is one of the actions associated with the herbicide in eudicots (CHINALIA *et al.*, 2007; SONG, 2014; GOGGIN *et al.*, 2016). The use of auxinic herbicides in high concentrations causes the activation of a series of steps in plant tissues. First, it stimulates metabolic processes, such as ethylene biosynthesis, followed by symptoms of dysregulated growth, such as tissue swelling in response to cell elongation and division. In the process of callogenesis, 2,4-D regulates plant growth in the cell as it stimulates the initiation of

cell division and growth by production of RNA polymerase, RNA and proteins (PINTO *et al.*, 2016). 2,4-D probably caused callogenesis in seeds owing to the first stage of action of auxinic herbicides that leads to tissue necrosis. The second stage occurs by inhibiting root growth, stomatal closure and production of reactive oxygen species (ROS). ROS are related to the oxidative process that causes tissues to darken (GUPTA, 2010). In the third stage, the disruption of cell membranes occurs, causing wilting, followed by necrosis and cell death (KELLEY AND RIECHERS, 2007; GROSSMANN, 2009).

Callogenesis was observed in seeds placed in vessels containing washed sand with 20, 40, 250 and 500 mg/L of 2,4-D. When germinating, callus was already observed in the region of the root axis and in the cotyledon. This fact is curious because reports of callogenesis are rarely found in plants submitted to treatment with 2,4-D herbicide in the field (ZIMDAHL, 1999; YAMASHITA *et al.*, 2009). The callogenesis response may be associated with the way 2,4-D is applied directly to the soil and its concentrations, although field application is mainly performed on foliage (SOUZA *et al.*, 2012). Regarding the contact of 2,4-D with seeds, the literature can be conflicting. That is, some studies prove that the herbicide is capable of altering the physiological quality of seeds, such as wheat, resulting in increased speed and total percentage of germination, while accumulating in the grain, even in concentrations below the maximum permitted limit (AISENBERG *et al.*, 2018). On the other hand, another study reported that wheat seeds pretreated with 10 μ M 2,4-D for 48h induced antioxidant activity and protective effect against salt stress (MOHSIN *et al.*, 2020). Studies with bean seeds, however, observed that 2,4-D does not have a stimulating effect, even at sub-doses, on seedling emergence and development since seeds treated with 33.50 g/L of 2,4-D had their mass indexes reduced by more than 70%. Moreover, after the application of 67 g/L of 2,4-D in pre-emergence, an injury rate of over 80% was noted (MASSUCATO *et al.*, 2021).

We observed that callus formation begins mainly in the region where the radicle emerges, an area of high cell production, where seed contamination also began. Seeds are rich in meristematic and active growth tissues, and since 2,4-D acts on cell division and the metabolism of nucleic acids and proteins, it also damages cell membranes and causes cell death, which explains why seeds are more responsive to auxin (MORO *et al.*, 2021). Massucato *et al.* (2021) also observed damage to the radicle of bean seeds, but they did not correlate with microbial proliferation as occurred in our experiment. In general, although little studied during the germination phase, microbial populations in the soil, substrate, or even endophytes, are critically important during the seedling emergence stage. They influence seed vigor, and they cause the production of antibodies and allelochemicals to inhibit plant fungal and bacterial pathogens, as well as induce systemic resistance and quorum quenching (OLANREWAJU *et al.*, 2017; HU *et al.*, 2018). The isolation and identification of microorganisms that colonized the seeds was not carried out in the present study. However, it is likely that the toxicity mediated by 2,4-D was

reflected in morphoanatomical, physiological and biochemical responses of these seeds, consequently inducing cell death and favoring the development of hemibiotrophic and necrotrophic microorganisms or early contamination of soil pathogens.

Riparian forest soil, as a substitute for sand and vermiculite, was taken from the Guandu River, which is located in the Baixada Fluminense region. Riparian forest are formations that occupy the margins of water courses (AB' SABER, 2004) that suffer anthropic actions, such as soil pollution through agriculture, pasture, and urban-industrial activity.

The use of sand was an advantage as it reduced interference in the results with soil composition, minerals and microorganisms. However, by using seeds from riparian forest areas, we could determine the most promising concentrations. The highest concentrations (250 and 500 mg/L) were discarded for presenting reduced germination and the lowest (20 mg/L) because the results showed no major differences in the percentage of germination with the 40 mg/L concentration of 2,4-D. The total number of seeds used in this stage was $n = 10$. The germination of *C. ensiformis* seeds was observed in the treatments without 2,4-D (Table 6) and a percentage of 20% in washed sand. No germination occurred in the condition of 40 mg/L of 2,4-D in sand substrate. Using sterile riparian forest substrate, seed germination was also observed at 30% without addition of 2,4-D after 4 days of sowing. Germination occurred only in sterile riparian forest substrate using 40 mg/L of 2,4-D (Table 6). The GVI results demonstrate that the control seeds with sterile riparian forest substrate were more vigorous compared to seeds of the same substrate with the presence of 2,4-D (40 mg/L) and sterile sand substrate (Table 6).

Table 6 - Germination of *Canavalia ensiformis* seeds in different substrates with and without 2,4-D, during 4 days.

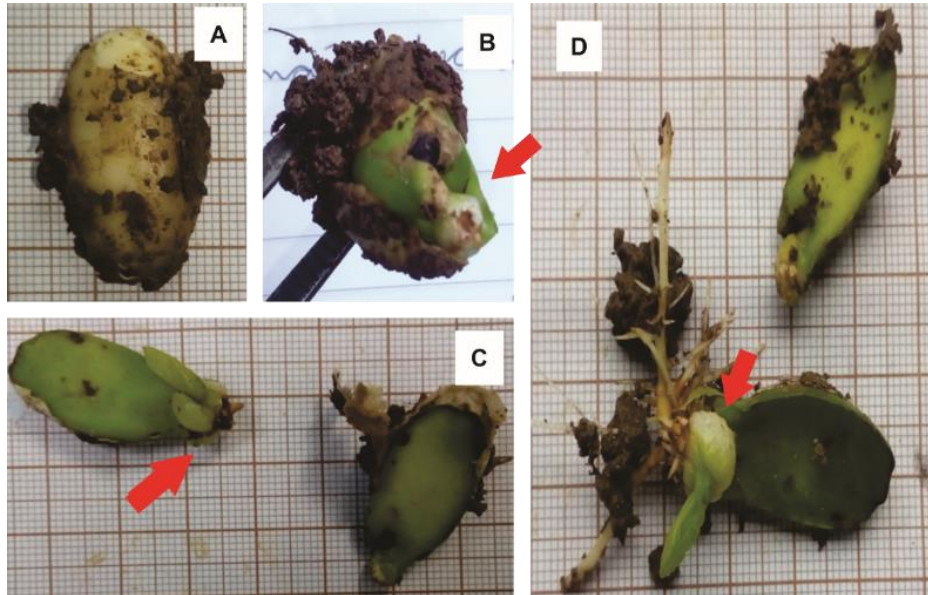
Substrates	Germination (%)			
	Control	GVI	2,4-D (40 mg/L)	GVI*
Washed sand sterile	20 ^a	0.5	0 ^a	0
Riparian forest not sterile*	0	0	0 ^a	0
Riparian forest sterile	30 ^a	0.75	20 ^a	0.5

*GVI= Germination Velocity Index. *From Guadu River. No significant difference was observed. Values in the row followed by the same letter do not differ according to the Tukey test, $n = 10$, $p < 0.05$.

Considering the initial development of *C. ensiformis* seedlings, the seeds sown in the substrate of the riparian forest of the Guandu River at a concentration of 40 mg/L of 2,4-D developed taproots that reached 3.5 cm on the 13th day after sowing (Figure 8D). On the 7th and

13th days, it was possible to observe a structure of cells with undifferentiated growth in the region of radicle development, indicating the formation of callus (Figure 8B, C and D).

Figure 8 - Seed with riparian forest substrate not sterile with 2,4-D (40 mg/L). A, B, C, D, respectively, 4th, 7th, 10th and 13th day after sowing of *Canavalia ensiformis* kept in a PET vessel. Red arrows indicate callus.



In this analysis, germination was clearly favored in sterilized versus non-sterilized substrates, reaffirming the importance of eliminating pathogens at the beginning of germination. However, it is important to note that nitrogen-fixing bacteria live in symbiosis with the roots of *Canavalia ensiformis*, and with the growth results of controls (without 2,4-D) in assay 2, it is possible to affirm that the sterilization of the substrates did not interfere with this relationship. The best germination results, GVI, and development were at a concentration of 40 mg/L of 2,4-D with sterile riparian vegetation substrate. The sowing process in riparian forest regions can be considered as an advantage compared to the application of seedlings because the process is less costly with no need for uniformity in growth or contact with contaminated substrate. The results indicate that this species presents better development in riparian forest substrate in which, even with the formation of callus, no early contamination of soil pathogens occurs. Therefore, it allows root growth owing to the presence of nutrients.

CONCLUSION

According to our experimental conditions, *C. ensiformis* is able to germinate in substrates heavily contaminated with 2,4-D. However, such germination is more prominent in vigorous seeds, as well as dependence on the use of riparian forest substrate at concentrations of up to 40

mg/L of 2,4-D. Both formation of calluses and enhanced microorganism proliferation were also observed with the methodology employed. This species presents better development in riparian forest substrate in which, even with the formation of callus, no early contamination of soil pathogens occurs, thus allowing root growth owing to the presence of nutrients. In summary, *C. ensiformis* did not demonstrate capacity as a phytoremediator since 2,4-D exposure resulted in damage to the plant's early development, preventing epicotyl, hypocotyl and leaf formation.

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