In vitro development of green manures: phytotoxicity and remediation of 2,4-D

Desenvolvimento in vitro de adubos verdes: fitotoxicidade e remediação de 2,4-D

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ABSTRACT

Evaluation of phytoremediation capacity under aseptic, controlled conditions can be achieved with the use of plant tissue cultures as a model, eliminating interference from the physical environment. Herein, we developed an *in vitro* culture protocol for green manures of *Crotalaria juncea*, *C. spectabilis* and *Canavalia ensiformis*, each of which contributes to a sustainable cropping system. In particular, we studied their behavior in the presence of the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Disinfection of *C.*

juncea seeds was efficient, and plantlets presented the best development of roots, stem and leaves. Thus, subsequent experiments were conducted only with *C. juncea*. For 2,4-D tolerance tests, *C. juncea* seedlings were transferred to Murashige and Skoog (MS) solid media under the following concentrations of 2,4-D: 0 (control), 0.2, 0.5 and 1.0 mg L^{-1} for a 30-day period. After this period, MS culture media for *C. juncea* was analyzed by HPLC for quantification of residual 2,4-D. *C. juncea* development was observed to be sensitive to 2,4-D toxicity; however it was observed 2,4-D reduction in the media. Nonetheless, field assays are still necessary to evaluate the feasibility of using it as a phytoremediator, as well as determine the influence of environmental and plant density variables on 2,4-D absorption capacity from soil.

Keywords: herbicide, bioremediation, pesticide, plant tissue culture, 2,4-D, green manures, phytoxicity, remediation

RESUMO

A avaliação da capacidade de fitorremediação em condições assépticas e controladas pode ser realizada com o uso de culturas de tecidos vegetais como modelo, eliminando a interferência do meio físico. Aqui, desenvolvemos um protocolo de cultivo *in vitro* para adubos verdes de *Crotalaria juncea*, *C. spectabilis* e *Canavalia ensiformis*, cada um dos quais contribui para um sistema de cultivo sustentável. Em particular, estudamos seu comportamento na presença do herbicida 2,4-D (ácido 2,4-diclorofenoxiacético). A desinfecção das sementes de *C. juncea* foi eficiente, e as plântulas apresentaram o melhor desenvolvimento de raízes, caule e folhas. Assim, experimentos subsequentes foram conduzidos apenas com *C. juncea*. Para os testes de tolerância a 2,4-D, mudas de *C. juncea* foram transferidas para meios sólidos Murashige e Skoog (MS) sob as seguintes concentrações de 2,4-D: 0 (controle), 0,2, 0,5 e 1,0 mg L-1 para um período de 30 dias. Após esse período, o meio de cultura MS para *C. juncea* foi analisado por HPLC para quantificação do 2,4-D residual. Observou-se que o desenvolvimento de C. juncea é sensível à toxicidade do 2,4-D; porém observou-se redução de 2,4-D na mídia. No entanto, ensaios de campo ainda são necessários para avaliar a viabilidade de seu uso como fitorremediadora, bem como determinar a influência de variáveis ambientais e de densidade de plantas na capacidade de absorção de 2,4-D do solo.

Palavras-chaves: herbicida, biorremediação, pesticide, cultura de tecidos vegetais, 2,4-D, adubos verdes, fitotoxicidade, remediação

1 INTRODUCTION

Studies have reported the use of green manures from the Leguminosae (Fabaceae) as a trend in phytoremediation. The process of green manuring involves growing crops ultimately incorporated into the soil to improve it and benefit subsequent crops. Indeed, the benefits of nitrogen fixation by bacteria in association with roots of green manures are already well known. This feature represents an additional step to exploit the phytoremediation capacity of target pollutants like pesticides (Foucault et al. 2013; Fumagalli et al. 2014; Donatti et al. 2017). Moreover, *C. juncea* is itself a weed suppressor through the mechanism of interspecific competition (Timossi et al. 2011).

Research reporting on the use of green manures, such as *Canavalia ensiformis* (L.) DC and *Crotalaria juncea* L., shows their potential in the removal of herbicides, such as sulfentrazone, from the soil (Oliveira et al. 2014; Belo et al. 2016; Ferraço et al. 2017) and *C. ensiformis* in the removal of 2,4-D herbicide (Silva et al. 2019); Phaseolus vulgaris L. in phytoremediation studies of the herbicide atrazine (Madariaga-Navarrete *et al.*, 2017). Still another study demonstrated that *C. juncea* and *Crotalaria spectabilis* were tolerant to the herbicides glyphosate, methylsufuron and 2,4-D in a mixture with glyphosate (glyphosate 1.800 $g.ha^{-1}$ + 2,4-D 2.010 $g.ha^{-1}$) (Concenço & Silva, 2015). The mineralization process of 2,4-D was observed on the rhizosphere of *Trifolium pratense* L. (Fabaceae) and *Lolium perenne* (Poaceae), indicating its specificity to plant species (Shaw & Burns, 2004). That some green manures are tolerant to this herbicide and adapted to the tropical climate may be a promising indicator for their use in the phytoremediation of 2,4-D, combined with the ability of these plants to contribute to nitrogen fertilization of the soil (Schultze-Kraft *et al.*, 2018).

Agrochemicals, such as 2,4-D (2,4-dichlorophenoxyacetic acid), are widely used in Brazil for their low cost (Chen et al., 2018; Nguyen et al., 2018). This herbicide can be selectively applied to both pre-emergence and post-emergence weed plants of the Eudicots, which may be present in wheat, soybean, corn, rice, sugarcane and pasture crops. It can be applied in a concentration range from 0.4 $L.ha^{-1}$ to 3.5 $L.ha^{-1}$ (DMA® 806 BR Dow Agrosciences). This corresponds to approximately 0.2 to 2.5 mg L^{-1}, depending on the invasive plant species and its degree of tolerance to the herbicide. When applied incorrectly, or under inappropriate atmospheric conditions, this herbicide can be carried by water (carryover), or even by wind (drift), up to 20 or 30 km away from the point of origin, eventually reaching other plantations, such as vines, and potentially causing damage to these neighboring sensitive plants and harming ecological balance. (Silveira, 2021). The use of 2,4-D due to the auxin effect is also recurrent in different plant tissue culture strategies (Costa *et al.*, 2020). In addition to its carcinogenic potential, exposure to 2,4-D can cause ocular damage to the liver and kidneys, central nervous system damage and dysregulation of the endocrine and reproductive systems (Jeffries *et al*., 2016; Nguyen *et al*., 2018). According to the package insert for 2,4-D (DMA® 806 BR, Dow Agrosciences), the half-life of the product is 4 to 10 days; but based on the review of the Baumgartner et al. (2017), it may vary from seven days up to several years. Among other factors, soil characteristics are directly related with the fate of 2,4-D and its bioavailability (Baumgartner et al., 2017; Jote, 2019; Meftaul et al., 2020). An important

fact to be consider is the influence of 2,4-D on the crop introduced after it application. In some cases, 2,4-D residues may influence the plant development until 40 days after application of the herbicide and up to 280 days when associated with other pesticide (Anésio et al., 2018). Another problem, is that 2,4-D can be persistent in the environment by accumulation throughout the food chain (Marcato et al., 2017). Thus, it is essential provide an efficient method to remediated 2,4-D herbicide.

Phytoremediation involves the use of plants to extract and remove elemental pollutants or lower their bioavailability in substrate (Yan *et al*., 2020). The establishment of plants for phytoremediation contributes to the reduction, degradation and/or stabilization of agrochemicals that pollute the general environment. Phytoremediation is considered cheaper than other remediation processes and can be applied on a large scale (Tarla *et al*., 2020). At the same time, microbes within the root zone may accelerate the degradation of organochlorine pesticides (OCPs) like 2,4-D (Singh & Singh, 2017). In phytoremediation studies, plant tissue culture is a valuable tool for assessing the plant's actual tolerance to the toxicity of the pollutant studied without the interference of microorganisms and abiotic stresses (Doran, 2009; Delmail *et al*., 2013; Koźmińska *et al*., 2018; Costa *et al*., 2019).

Therefore, this work aimed to 1) evaluate the *in vitro* development of *C. ensiformis*, *C. spectabilis*, and *C. juncea*, the most applied green manures in Brazil (Espíndola *et al*., 2005; Ambrosano *et al*., 2016), under different 2,4-D concentrations; 2) evaluate morpho-physiological responses of green manures in the presence of 2,4-D; and 3) analyze the concentration of 2,4-D left in the culture medium after plantlet development.

2 MATERIALS AND METHODS

2.1 SEED DECONTAMINATION

Seeds of *Crotalaria juncea*, *C. spectabilis* and *Canavalia ensiformis* were purchased commercially at the Futuro Fértil Store - (S. T. Irajá Agricola Ltda) located in the State Supply Centers (Ceasa-RJ). All seeds belonged to the 2015 crop, lot 08/2015, with a purity of 98% and a germination rate of 75%. *Crotalaria juncea* and *C. spectabilis* seeds were disinfested by immersion under agitation for 15 min in sterile water with detergent and then immersed under agitation for 10 min in sodium hypochlorite (2.5%). Finally, the seeds of these two species were then washed for 1 min in 70% ethanol and then rinsed three times with sterile distilled water. *Canavalia ensiformis* seeds were

soaked in distilled water for 30 min, maintained under agitation for 7 min in sterile water with detergent, and then immersed for 10 min in sodium hypochlorite (2.5%). Then, the seeds were washed for 1 min in 70% ethanol and rinsed three times with sterile distilled water.

2.2 GERMINATION AND SEEDLING DEVELOPMENT

Four seeds each from *C. juncea* and *C. spectabilis* and one seed from *C. ensiformis* per glass were introduced in a total of 20 glasses in 10 repetitions for each species. To each glass were added 50 mL of MS (Murashige & Skoog 1962) nutrient medium containing 3% sucrose and 0.6% agar (pH 5.6–5.8). Cultures were maintained in a growth room under a photoperiod of 16 h light/8 h dark, as provided by white fluorescent tubes (light intensity of 5.616 $\mu mol.m^{-2}.s^{-1}$) and temperature of 25±2°C.

The developmental parameters analyzed were germination (%) and, after germination, plantlet height (cm), number of leaves, rooting (%) and root length (cm). Such analysis was carried out on the 30th day post-germination.

2.3 EVALUATION OF CROTALARIA JUNCEA DEVELOPMENT AND REMEDIATION OF 2,4-D

At 30 days post-emergence, the seedlings were transferred to MS media containing different concentrations of 2,4-D (plant cell culture tested, > 98% purity, Sigma-Aldrich®): 0 (MS0-control), 0.2, 0.5 and 1.0 mg L^{-1}. Such concentrations were established for this experiment because the concentrations used in the field are between 0.2 and 2.5 mg L^{-1}, and the recommended dosage of 2,4-D for most application situations is 1.5 $L.ha^{-1}$, equivalent to approximately 1.07 mg L^{-1} (DMA® 806 BR Dow Agrosciences). The 2,4-D we used is a pure product, not the commercial herbicide which, in the presence of adjuvants, could interfere with the first analysis of plant growth in the culture medium. The experiment was conducted with two seedlings per glass in 10 repetitions for each concentration of 2,4-D. The culture conditions were the same as those described above. At the end of the 30th day, plant development observations were made based on the same parameters as those noted above in order to determine any possible changes in the morphology of the seedlings in the presence of 2,4-D. Callogenesis, for example, was observed by the presence of *calli* in some cultures. For an evaluation of the remediation of 2,4-D, an indirect analysis was made by considering residual 2,4-D concentration in the medium after 30 days of cultivation of the green manures. The media

were processed for further HPLC (High-performance Liquid Chromatography) analysis as described in the next section.

2.4 ANALYSIS OF CULTURE MEDIA BY HPLC

Aliquots of the residual media from each treatment were stored in sterile test tubes at 4°C until the time of processing for analysis by HPLC. Sample preparation of all culture media for analysis consisted of acidification with concentrated HCl (2.5 µL of acid for each mL of medium), followed by autoclaving at 121°C at 1 atm for 15 minutes (Costa et al. 2020). After cooling the media, the samples were filtered (0.22 µm pore filters) into vials and held at -20°C until analysis. Chromatographic conditions were those described by Costa et al. (2020). For quantification of 2,4-D in culture media, a Zorbax Eclipse XDB-C8 chromatographic column 150 mm long by 4.6 mm in diameter in stationary phase (reversed phase) was used with irregular granules 5 µm in diameter. Also used was an Eclipse XDB-C8 pre-column 12.5 mm long by 4.6 mm in diameter with irregular granules 5 µm in diameter on the Agilent 1260 Infinity Series chromatograph. Detection of 2,4-D was performed at 225 nm. Data acquisition and processing were performed using the Agilent ChemStation program. The mobile phase consisted of ultrapure water acidified with 0.1% (v/v) phosphoric acid and acetonitrile, also acidified with 0.1% (v/v) phosphoric acid. In the first 3 min, the acetonitrile concentration was 30%. From 3 min to 10 min, it ranged from 30% to 100%, and from 10 min to 15 min, it was maintained at 30%. The time of the chromatographic run was 15 min for each sample with 2,4-D detected at the retention time (RT) of 8 min. The oven temperature was 45°C, and the flow rate at each injection in this method was 1 $mL.min^{-1}$. The injection volumes used were 80 µL. The calibration curve was calculated as $y = 101.1x + 13.133$, the linearity of which was $R^2 = 0.9967$ for a limit of detection (LOD) = 0.0967 mg L^{-1} and a limit of quantification (LOQ) = 0.3224 mg L^{-1}. The analysis was performed in quintuplicate.

2.5 STATISTICAL ANALYSIS

BioEstat 5.0 software was used to analyze the plantlets' morphological parameters (dry weight, number of leaves, stem length and root length), as well as quantify the residual 2,4-D present in the MS culture medium after culturing C. juncea. ANOVA was

used, adopting a value of $p < 0.05$ as a significant result. One-way ANOVA was followed by Tukey's multiple comparisons test.

3 RESULTS AND DISCUSSION

3.1 SEED GERMINATION AND DEVELOPMENT OF SEEDLINGS

Seed decontamination and germination were 100% efficient for *C. juncea* and *C. spectabilis*, which germinated on the 2nd day of culture, reaching 100% germination. After 30 days, *C. spectabilis* presented root length of 4.6±0.2 cm, number of leaves of 3.4±0.2, and root length of 5.4 cm±0.2. *C. juncea* presented the best development with stem length of 10.8±2.6 cm, number of leaves of 6.4±0.2 cm, and root length of 6.0±0.2 cm (Figs. 1, 2). For *C. ensiformis*, 67% of the seeds were contaminated with only one seed germinating on the 3rd day of culture. The single germinated plantlet of *C. ensiformis* presented a single root of 2 cm, 4 leaves and 4.5 cm of height after 30 days of culture. For this reason, our studies with *C. spectabilis* and *C. ensiformis* were dropped since the developmental parameters of *C. juncea* were judged to be the best of the three species evaluated.

Figure 1. *In vitro* seed germination and seedling development of *Crotalaria juncea*. A) Cultured seed type on solid MS0 (control) medium containing 3% sucrose after two days of cultivation, showing the radicle (black arrows), Bar= 0.81 cm; B) 21st day after germination in MS0; plantlets with leaves and roots, reaching the top of glass, Bar= 3.2 cm; C) Subcultures of *C. juncea* after 30 days in MS0, 75% rooting. Visible morphological alteration in leaves (eclipse), Bar= 2.38 cm; D) 30 days of subcultures in MS + 0.5 mg L^{-1} of 2,4-D: white arrow indicates the presence of calli and quadrate new shoots in the base of plantlets, Bar= 1.81 cm; E) 30 days of subcultures in MS + 0.2 mg L^{-1} of 2,4-D: narrowing of the stem (stem damping off) (white arrow) and calli at the base of plantlets (white arrow), Bar= 2.63 cm; F and G) 30 days of culture in MS + 1.0 mg L^{-1} of 2,4-D: narrowing of the stem and adventitious roots (white arrow) (F, Bar= 0.53 cm and G, Bar= 6.35 cm).

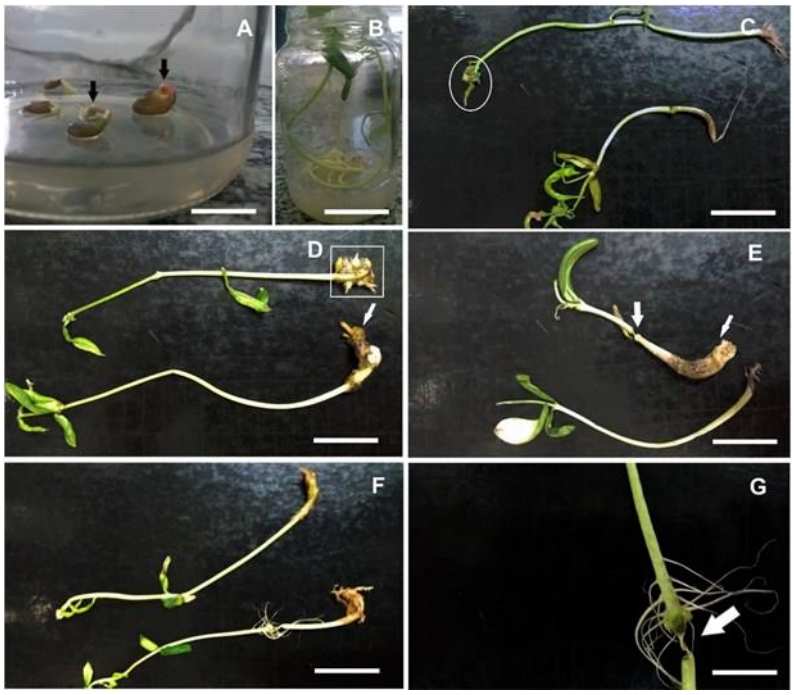
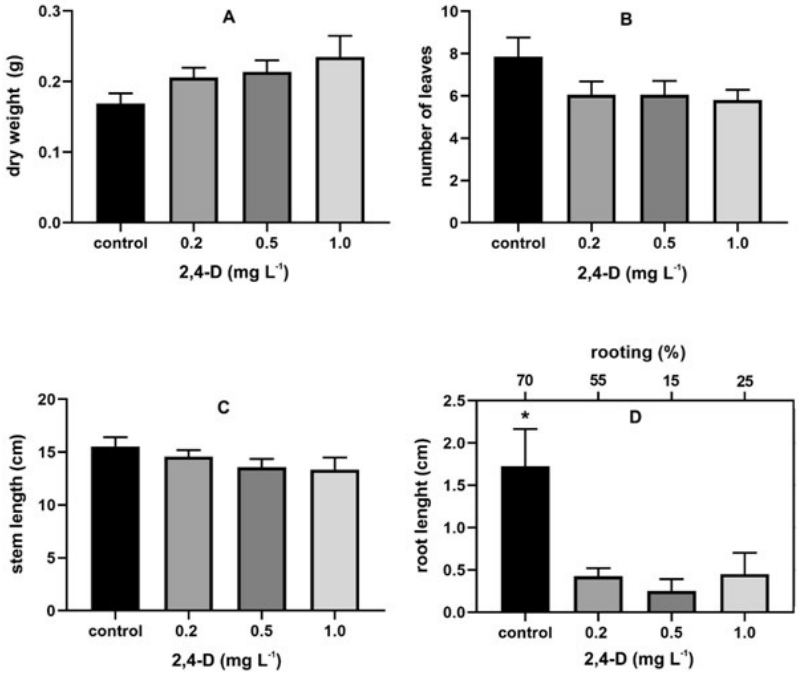


Figure 2. Morphological performance of *Crotalaria juncea* under different concentration of 2,4-D, (control:MS0, 0.2, 0.5, 1.0 mg L^{-1}): A. dry weight of plantlet, B. number of leaves of plantlets, C. stem length and D. root length, n= 20, after 30 days of culturing. Bars indicate the standard error. No significant differences were observed for parameters; significant difference ($p< 0.05$) assessed by one-way ANOVA followed by Tukey's multiple comparisons test. **Rooting percentage above 1.0 cm.



3.2 EVALUATION OF THE DEVELOPMENT OF *C. JUNCEA* PLANTLETS UNDER 2,4-D ADDITION

Figs. 1 and 2 show the development of plantlets submitted to 2,4-D concentrations: (control, MS0), 0.2, 0.5, and 1.0 mg L^{-1} after 30 days. The morphological performance of *C. juncea* under 2,4-D treatments presented increasing dry weight along increasing concentration, albeit not statistically significant ($p<0.05$) (Fig. 2A). Approximately10% of seedlings cultured in the MS medium plus 0.2 mg L^{-1} of 2,4-D, or 0.5 mg L^{-1} of 2,4-D (Fig. 1D), presented callus-like protuberances at the base of the stem and small shoots in some plantlets (Fig. 1E).

In all *C. juncea* seedlings treated with 1.0 mg L^{-1} of 2,4-D (Figs. 1F, 1G), we noticed a narrowing in some portions of the stem that resembled stem damping off as a result of some soil-borne fungal disease. When this occurred, adventitious roots appeared in the upper part of this stem, causing the characteristic narrowing noted above.

At the 30th day of culture in MS0 medium, the rooting percentage reached 70% (Figs. 1B, 1C, Fig. 2D). In contrast, seedlings cultured in media containing 0.5 mg L^{-1} and 1.0 mg L^{-1} of 2,4-D showed damage in the formation of roots, resulting in only 15 and 25% rooting, respectively (Figs. 1D, 1G, Fig. 2D).

The mean values of stem length (Fig. 2C) and number of leaves (Fig. 2B) of plantlets cultured in media without 2,4-D were the highest in comparison to all concentrations of 2,4-D tested (0.2, 0.5 and 1.0 mg L^{-1}). These results indicate that 2,4-D caused damage to the development of *C. juncea* from the concentration of 0.2 mg L^{-1} forward, probably by 2,4-D toxicity, as manifested in the decrease in root length, percentage of rooting, plantlet height and number of leaves compared to control.

After cultivating the *C. juncea* seedlings for 30 days in media with different concentrations of 2,4-D, an analysis of variance was performed to determine residual 2,4-D in the media, but no significant statistical differences ($p <0.01$) were observed between the 0.5 and 1.0 mg L^{-1} 2,4-D concentrations (Tab. 1). In samples originally containing 0.2 mg L^{-1} of 2,4-D, it was not possible to detect 2,4-D (Tab. 1).

Table 1. Initial and final concentrations (IC and FC) of 2,4-D (mg L^{-1}) for treatments performed in solid MS medium with *in vitro* cultures of *Crotalaria juncea*.

IC (mg L^{-1})	**FC after *C. juncea* transplant (mg L^{-1})**	**Reduction of 2,4-D (%)**
MS0-Control	nd	nd
0.2	nd	100
0.5	0.38±0.3	24
1.0	0.68±0.3	32

Data (Mean±SD) obtained by HPLC analyses. *After culture of *Crotalaria juncea* for 30 days; nd: not detected (≤0.096 mg L^{-1}); n= 20. IC – initial concentration. FC – final concentration.

Tab. 1 shows the initial and final concentrations of 2,4-D in the MS media for all treatments performed. The concentrations obtained for each treatment at the end of 30 days were below the LOD (≤ 0.096 mg L^{-1}) in the MS0 medium, and the same result was found for the final 2,4-D concentration when its initial concentration was 0.2 mg L^{-1}(Tab. 1). In media with initial concentrations of 0.5 mg L^{-1} and 1.0 mg L^{-1} of 2,4-D, the concentrations of 2,4-D reported after 30 days were 0.38±0.3 mg L^{-1} and 0.68±0.3 mg L^{-1} of 2,4-D, respectively.

C. juncea was able to absorb all 2,4-D available in the culture medium that was initially prepared with 0.2 mg L^{-1} (Tab. 1). However, in media prepared with 0.5 mg L^{-1} of 2,4-D, the reduction of the herbicide in the medium was 76%, whereas in the medium with 1.0 mg L^{-1} of 2,4-D, the reduction was 68%. However, considering the margin of error and statistical analyses, these reductions were not significant.

4 DISCUSSION

4.1 GERMINATION AND SEEDLING DEVELOPMENT

Tissue culture is associated with physiological studies of plant development and metabolism, clonal cleaning or genetic transformation. Only a few studies have reported on the plant tissue culture of *C. juncea*, possibly because green manures tend to develop very well in the field. Indeed, no in-depth studies reporting on *in vitro C. juncea* development can, to the best of our knowledge, be found in the literature. Rocha and Campos (2004) did study the *in vitro* culture of *C. juncea*, but the aim was to determine callus production in order to evaluate the effects of plant exudates on the inhibition of a phytonematode.

C. juncea seeds germinated rapidly for up to 2 days in sterile conditions. Within five days after germination, *C. juncea* plantlets approached the top of the glass, while roots occupied the entire basal area in the culture medium inside the glass.

The development of plantlets nearly stagnated after the twenty-first day for all culture media, with or without 2,4-D. This could be attributed to the *in vitro* conditions in which nitrates may not be present in the amount required for *C. juncea*. Under field conditions, however, the symbiotic relationship between nodules and bacterial colonies present in legume roots leads to biological nitrogen fixation (BNF) in the form of nitrate, a macronutrient essential for plants (Madalão *et al*., 2016).

4.2 RESPONSES OF *CROTALARIA JUNCEA* CULTURE UNDER 2,4-D ADDITION

Toxicity responses were observed in seedlings developed in the presence of 2,4-D, as manifested in twisting of stems and brittleness. 2,4-D is a well-known non-selective herbicide for broadleaf Eudicots that kills them by altering the plasticity of their cell walls, affecting protein synthesis, and even by increasing plant ethylene production, causing stems to curl over, leaves to wither, and plants to die (Song, 2014). Therefore, its use seems to affect green fertilizers, but also adjacent plantations by the risk of accidental risk drift (Silva *et al*., 2018).

As we showed in our results, calli were formed. This may be a morphological response associated with the auxinic effect of 2,4-D. Studies have reported on callogenesis in plant cultures subjected to 2,4-D at concentrations that may vary from 0.0045 mg L^{-1} to 10 mg L^{-1}, depending on the species cultured (Trigiano & Gray, 1999; Conger 2018). Nevertheless, the results still suggested the possibility of performing remediation with *C. juncea* for the removal of residual 2,4-D in the MS culture media and for use in *in situ* phytoremediation.

Data of *C. juncea* dry weight showed increased values from control medium (0.17 g) to 1.0 mg L^{-1} (0.24 g), but with no statistically significant difference. Single-plant dry weights indicate plant growth ability (Wang *et al*., 2017). However, considering other morphological parameters, increasing 2,4-D concentrations resulted in a reduction in this value (Fig. 2), likely indicating changes in plant tissue features and/or metabolism. In general, tissues treated with auxins have a higher water content, possibly from cellular stretching promoted by auxin acidification in the cell wall (Dang *et al*., 2020). The fresh weight-to-dry weight ratio is a well-known indicator for cell viability because higher ratios mean more chance for culture viability (Fu *et al*., 2005). Thus, since the values did not differ significantly from the control, no variation in cell viability occurred based on dry weight.

Interestingly, during the experiments, shoots formed in plantlets submitted to concentrations of 0.2 and 0.5 mg L^{-1} of 2,4-D by 55% and 15%, respectively. Under MS0 media, no shoots were identified. With addition of 1.0 mg L^{-1} of 2,4-D, the presence of stem narrowing, similar to damping off (Lamichhane *et al*., 2017), occurred, together with emerging shoots and adventitious roots in 20% of plants. The absorption of the herbicide also resulted in changes in development, such as callus formation and twisted stems. Otherwise, 2,4-D in low doses promotes plant growth in different *in vitro* cultures. On

the other hand, at high doses, it can drive plant overgrowth (Song *et al*., 2014). Such overgrowth is expressed through cupping and stunting of leaves, brittleness, stunting and twisting of stems, as well as general abnormal growth (Grossmann, 2009). Plant regulators like 2,4-D can cause enhanced DNA ploidy levels and methylation events in plant tissue culture (Bidabadi & Jain, 2020). In our experiments, we tried to mimic the concentrations commonly used in the field. However, the damping off appearance was also observed in the control experiment without 2,4-D, suggesting that the *in vitro* condition itself contributed to this response.

Damping off, as briefly cited above, is a physiological tipping in young plants caused by stress, such as the colonization of pathogenic microorganisms, e.g., fungi and bacteria, which can be favored in the field under desbalancing environmental conditions like high soil temperature and humidity (Lamichhane *et al*., 2017). However, it may be recalled that we started the experiment with an aseptic environment absent of microorganisms. It is possible that high humidity under *in vitro* conditions caused the damping off. This is the first study to report this behavior of *C. juncea* under *in vitro* conditions. The *in vitro* environment can be a stressor in its own right. Specifically, from culture initiation through acclimatization, plants are subjected to mechanical perturbations, air embolisms owing to dissection, poor nutrition, desbalancing in hormone treatments, high relative moisture in the culture flask (Van Staden *et al*., 2006), and medium composition and light regime that may induce plant epigenetic events (Bidabadi & Jain, 2020). In a comparison between “soil” and “culture medium”, whether solid or semi-solid, the culture medium represents a “compacted soil” with low porosity, making water movement and root development difficult and causing biotic stress to seedlings (Lamichhane *et al*., 2017).

4.3 REMEDIATION 2,4-D FROM MEDIA

It is important to note that assays to verify the absorption of 2,4-D from *C. juncea* culture media were performed by transferring the whole plant to a new medium. Choosing to use the whole plant ignores the developmental stage during which subculture of nodal segments is carried out in newly formed plantlets. As previously stated by Doran (2009), whole plants, or plant organs, have been used to evaluate studies of *in vitro* phytoremediation. (Talano *et al.*, 2010; Angelini *et al*., 2011).

Comparing initial and final concentrations of 2,4-D in the MS medium, it was observed that 2,4-D remediation by plantlets from media contained 0.2, 0.5 and 1.0 mg

L^{-1} at the end of 30 days of culture (Tab. 1). However, the absorption of 2,4-D was not maximal for *C. juncea* at 0.5 and 1.0 mg L^{-1} of 2,4-D. Based on the fresh weight-to-dry weight ratio, the decrease in the concentration of 2,4-D in the medium resulted from extraction by plantlets. Previous experiments ruled out the possible degradation of 2,4-D by light under *in vitro* cultures of *C. juncea*, confirming the remediation of 2,4-D by plantlets (Costa *et al*., 2019). Therefore, notwithstanding the negative effects of 2,4-D on the initial development of plantlets, 2,4-D remediation was still confirmed, as was its toxic effect, on *C. juncea* plantlets. In a sense, these results provide guidelines for the universal study of any given pollutant in the context of any given plant's intrinsic phytoremediation capacity under phytosanitary conditions.

From a broader perspective, the density of plants might influence physiological mechanisms responsible for phytoremediation because of the role played by the volume of the root system and the transpiration of plants by area. Such factors may result in optimized absorption of xenobiotics like pesticides. This capacity may be enhanced by rhizo-degrading microorganisms associated with plant roots (Ferraço et al. 2017). For this reason, phytoremediation programs usually implement densification practices with remediating plants up to a certain limit in order to speed up the process (Ferraço et al. 2019).

In sum, *C. juncea* did not develop satisfactorily under the proposed *in vitro* conditions. Nevertheless, we still saw a reduction in 2,4-D of 100%, 24% and 32% of 2,4-D in the culture medium (0.2, 0.5 and 1.0 mg L^{-1}, respectively) after 30 days. However, before claiming the use of this plant for 2,4-D phytoremediation, we asked whether the plant just degrades the pesticide or accumulates it. This is a critical point because green manures, after growth, are cut and left in the soil to provide nutrients for a given crop. If the plant only accumulates the pesticide, then the pollutant can just be recirculated back into the environment. Accordingly, this question opens an avenue of investigation to determine the actual process of phytoremediation in this plant species and the number of plants, as well as time required, to achieve 100% of 2,4-D in the field.

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