

## Effects of Bermuda grass and Alfalfa extracts on callus induction of *Rye embryo*

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### ABSTRACT

We investigated the effects of Alfalfa (*Medicago sativa* L.) and Bermudagrass (*Cynodon dactylon* L.) extracts concentrations on callus induction and rye (*Secale cereale* L.) embryo regeneration and growth of rye seedlings in both greenhouse and *in-vitro* conditions. Immature rye embryos were used as explants for generating callus, and water extracts of alfalfa and bermudagrass were added to the environment with a concentration of 5, 10, 15, 20, 25 and 30 %. This extract was also used in the regeneration period. The use of 25 and 30 % alfalfa extracts stopped callus induction and its components. The application of bermudagrass extract with a concentration of 30 %, reduced rye callus induction to 50 %. Alfalfa extract concentrations of 20 and 25 %, respectively caused a reduction of 35 and 75 % in callus fresh weight in comparison with the control conditions. Applying 25 and 30 % concentrations of bermudagrass extract led to a reduction of 68 and 80 % respectively. Alfalfa extracts with 15, 20 and 25 % concentrations caused reductions of 40, 63 and 69 % in rooting. The 30 % concentration of *M. sativa* extract stopped rooting in rye callus. Concentrations of 15, 20, 25 and 30 % caused reductions of 31, 50, 55 and 81 % in the shooting of rye callus, respectively. The results indicate that alfalfa extract had greater effect on rooting, and the bermudagrass extracts had greater effect on shooting. The analysis of the results shows that Alfalfa and bermudagrass extracts have a strong allelopathic effect, which can be used in the production of herbicides of natural origin.

**Keywords:** Alfalfa, Allelopathy, Bermudagrass, Callus, *Cynodon dactylon*, Extract, *Medicago sativa*, Rooting, Rye, *Secale cereale*, Shooting.

### INTRODUCTION

Rye (*Secale cereale* L.) is usually grown in colder climates such as Canada and Northern Europe, where wheat and barley cannot be grown (5). The area under rye cultivation is 4.4 m. ha and grain yields ranges from 2.0 to 5.8 mt ha<sup>-1</sup> especially in Northern Europe (4).

Allelopathy is a common biological phenomenon by which plants affect the growth, survival and reproduction of other living organisms by producing biochemical substances (21). These biochemicals are known as allelochemicals and have beneficial or harmful effects on target organisms (24). The allelopathic effects of plants is one of the modes of interaction between recipient or donor plants and may have beneficial effects (in agricultural

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management: weed control, product protection, product re-establishment), or harmful effects (self-poisoning, autotoxicity, causing soil diseases or have biological) (3).

Allelopathic compounds strongly inhibit mitosis and mess up the structures of organelles like the nucleus and mitochondria (13,19). Some allelopathic compounds prevent the transduction of polar auxin and cause irregularities in normal levels of auxin leading to the generation and inhibition of lateral roots. High levels of allelopathic compounds may result in reduced root growth because they inhibit the oxidation of auxin, which inhibits auxin's ability to induce ethylene synthesis in root tissues. High levels of auxin have an inhibitory effect on roots.(27,31). The allelopathic compounds affect the callus induction in tissue culture and embryogenesis (25). Al-Saleh *et al.* (3) evaluated the effects of neem and oleander extracts on sunflower callus cultures. Results showed that these two plants extracts with concentrations of 1.5 and 2.5 mg per ml stimulated the growth of callus. Abenavoli *et al.* (2) reported that metabolic and morphological changes in the leaf explants of *Petunia hybrida* are induced by Coumarin. The tissue cultured in the presence of Coumarin had more elongated roots with fewer hairy roots. The leaf explants cultured with Indole Acetic Acid (IAA) had more hairy roots but fewer roots. The presence of both IAA and Coumarin stimulated the production of more long and hairy roots. Auxin oxidase activity was low in cultures that had received Coumarin treatment, which suggests that Coumarin may regulate endogenous auxin levels by interfering with the peroxidase system. Manners and Galitz (20) reported that extract concentrations of 200 to 500 ppm of *Antennaria microphylla* has a negative effect on Spurge callus growth. Soybean and rice calluses were cultivated in the same culture in Yang and Futsuhara's (32) and Chon and Nelson's (9) studies. In this study, the allelopathic effect of rice was so severe that the growth rate of soybean callus was reduced 100 times in the experimental conditions. The effects of water extract and *A. princeps*' volatile components on the development of various species and callus induction were studied. *A. princeps* extract at 5 % inhibited callus growth. Despite the fact that these cells appeared to be normal, callus development was not prompted in the explants from various plants in high concentrations. Lettuce and *Eclipta prostrata* were very sensitive species. *A. princeps* was also impressed by its own extract (15,16). Kil *et al.* (17) reported that the extract of *Artemisia, lavandula efolia* reduced callus growth in rice, rapeseed, *Brassica campestris* and *Achyranthes japonica*. In Choya and Sharma's (11) experiment, allelopathic compounds of *Lantana camara* reduced regeneration in tissue culture. Regeneration decreased with increasing the concentration of the extracts. In this study, the effect of leaf extracts was more than that of other organs of the plant. Batisha *et al.* (6) reported that the carboxylic acid reduced mung bean root growth in explants and this compound reduced the amount of decrease by affecting the formation of the roots.

In light of the aforementioned information, the objective of this study is to investigate the effects of various concentrations of allelopathic extracts of alfalfa and bermudagrass on the induction and regeneration of calluses in rye.

## MATERIALS AND METHODS

The study was conducted in 2018 in Islamic Azad University Agricultural Station Tabriz , Iran (46° Longitude, 38° Latitude, height above sea level: 1360 m, Annual rainfall: 243.6 mm, a maximum and minimum temper was 28.25 and 10.57, respectively.

**Plant material and growing conditions:** This study was carried out, using a factorial experiment based on a completely randomized design (CRD) with 3 replications. To use immature embryos as explants in the tissue culture of rye, rye seeds were first cultivated in a greenhouse in 9-liter pots. 10 to 18 days after pollination, the spikes were harvested, and the produced grains were removed and disinfected. To disinfect them, at first, they were put in 70 % alcohol and 0.1 %  $\text{HgCl}_2$  for 15 min and then in 5 % Sodium hypochloride solution for 20 seconds, followed by a 5 times sterilization in distilled water (33). Afterwards, the immature embryos were isolated from the grains and put in the previously-prepared culture medium for callus induction (Figure 1).



Figure 1. The recipient plant, Rye (*Secale cereale* L.)

In this study, the culture medium was of MS type, which contained macro and micro-nutrients, Myoinositol, vitamins, carbon sources,  $1.0 \text{ mg L}^{-1}$  6-BA and  $0.7 \text{ mg L}^{-1}$  NAA and agar. All these steps were conducted under a laminar hood and in sterile conditions. The MS was supplemented with a range of  $(1.0 \text{ mg L}^{-1})$  2, 4-D (2, 4-Dichloro Phenoxy Acetic acid) in plastic culture bottles (240 ml, 10 cm in height and 7 cm in diameter) containing 33 ml of the medium. The culture medium containing 3.0 % sucrose was solidified with 0.75 % agar. For the induction of callus in embryos, they were placed on in the incubator at  $25 \pm 1 \text{ }^\circ\text{C}$  under fluorescent light ( $100 \mu\text{mol.m}^{-2}\text{s}^{-1}$ ) in a lighting regime of 12:12 h (light/dark) at  $25 \pm 1 \text{ }^\circ\text{C}$ , to observe the effect of 2, 4-D on callus subculture for 6 weeks. Every 3 weeks, the medium was replaced, and the culture was carried out again (8,29).

At the same time, to determine the allelopathic effects in the tissue culture, the allelopathic extracts from plant organs, which were in their developmental stages, were added to the culture medium to trigger callus production. To prevent infection, allelopathic extracts were initially passed through a 20-micron filter, and then they were used. The concentration of the added allelopathic extracts ranged between 5 and 30 % (5, 10, 15, 20, 25 and 30 % respectively). Bermudagrass (Figure 2) and Alfalfa (Figure 3) parts were harvested from fields in flowering stage and dried at  $60 \text{ }^\circ\text{C}$ ; then 20 g of dried residue was left floating in 100 cc distilled water for 24 h and centrifuged (10).



Figure 2. Bermudagrass (*Cynodon dactylon* L.)



Figure 3. Alfalfa (*Medicago sativa* L.)

The number of samples in each concentration of extract was 10 and the control included 100 samples. Thus, the total number of samples was 160. At the end of the 6-week period, the size, fresh weight and dry weight of the obtained callus in 5 samples out of the total number of 10 samples were calculated. In this way, the allelopathic effect of the extracts on callus induction was determined. The parameters analysed for evaluating the effect of the extract with the regeneration test by preparing 5 different concentrations including (5, 10, 15, 20, 25, 30 %).

**Regeneration test:** The prepared callus samples were put in control conditions for shoot regeneration for 30 days in MS medium containing ( $1.0 \text{ mg. L}^{-1}$ ) 6-BA in the growth chamber with 16 hours of light time (fluorescent light  $100 \mu\text{mol.m}^{-2}\text{s}^{-1}$ ) and 8 hours of dark period at a temperature of  $25 \pm 1 \text{ }^\circ\text{C}$ . For rooting medium, which consisted of quarter-strength MS medium supplemented with concentrations of NAA ( $0.1 \text{ mg. L}^{-1}$ ) for 20 days under fluorescent light ( $100 \mu\text{mol.m}^{-2}\text{s}^{-1}$ ) in a lighting regime of 16:8 h (light/dark) at  $25 \pm 1 \text{ }^\circ\text{C}$  (30). In order to determine the allelopathic effects of the extracts, the extracts with pre-defined concentrations were added to the culture medium. After the shoot and root apparition in callus, their weight and size were calculated. For all in vitro studies, the pH of the medium

was adjusted to 6.5 prior to the addition of agar; 33 ml medium was dispensed in glass growth vessels and autoclaved at 121 °C and 1.0 kg.m<sup>-2</sup> for 20 min.

(Analyzed parameters for regeneration assessment; Callus induction (%), callus volume, callus fresh weight, callus dry weight and Rye regeneration including Rooting (%), Root Length, Shooting (%), Shoot Length and Seedling length are explained in results section).

**Statistical analysis:** The experiments were conducted on the basis of Randomized Complete Design. All experiments were based on factorial experiment with 3 replications. The statistical test of ANOVA was used to analyze the data. The comparison of means was made using Duncan test (P<5 %), and the graphs were drawn using Excel software.

## RESULTS AND DISCUSSION

Analysis of variance of the traits showed that plant type, extract concentrations and their interactions had a significant effect on most of the traits (Table 1).

Table 1. Mean squares of evaluated traits in rye.

S.O.V.	df	Means square								
		Callus induction	Callus volume	Callus fresh weight	Callus dry weight	Rooting (%)	Root length	Shooting (%)	Shoot length	Seedling length
Plant	1	1037.7**	1.84**	0.199**	0.216**	85.26 ns	1.07 ns	1503.13*	1.71*	0.08 ns
Conc	6	4210.9**	6.44**	0.07**	0.089**	8467.13**	27.28**	5544.9**	16.38**	90.51**
Plant × Conc	6	759.2**	0.83**	0.086**	0.089**	141.47 ns	0.33 ns	186.15 ns	0.21 ns	0.53 ns
Error	28	65.7	0.15	0.005	0.005	165.84	0.589	237.01	0.53	1.217
CV (%)		15.63	16.51	6.53	2.15	22.46	24.52	24.69	26.85	19.16

\* and\*\*: significant at 5 % and 1 % levels, respectively, ns: non -significant

**Callus induction (%):** With 5 % (*M. sativa*) extracts, the percentage of callus induction of rye showed a significant increase of 19 % compared to the control conditions; that is, the treatment promoted the stimulation of callus induction (Figure 1). Mucciarelli *et al.* (23) reported that callus induction is stimulated under the influence of low concentrations of some plants extracts. Regulating the concentration of hormones such as auxin and gibberellin is also essential for normal cell growth and morphogenesis. A number of flavonoids can stop the polar auxin from being transduced, and auxin can cause abnormalities on the usual auxin surface, which can induce and inhibit lateral root geotropism. Benzoic acid is also involved in auxin catabolism (31). The percentage of Rye callus induction decreased with increasing concentrations of alfalfa extract. Also 25 and 30 % concentrations of alfalfa extract inhibited rye callus induction. (*C. dactylon*) extract with a concentration of up to 20 % did not have a significant effect on the percentage of callus induction, but in extract concentrations of 25 and 30 %, there was a decrease by 38 % and 50 % respectively. Simple linear regression showed that for every one- unit increase in the concentration of alfalfa extract, 16.497 units of alfalfa and 6.595 units of bermudagrass callus induction would reduce (Figure 4).

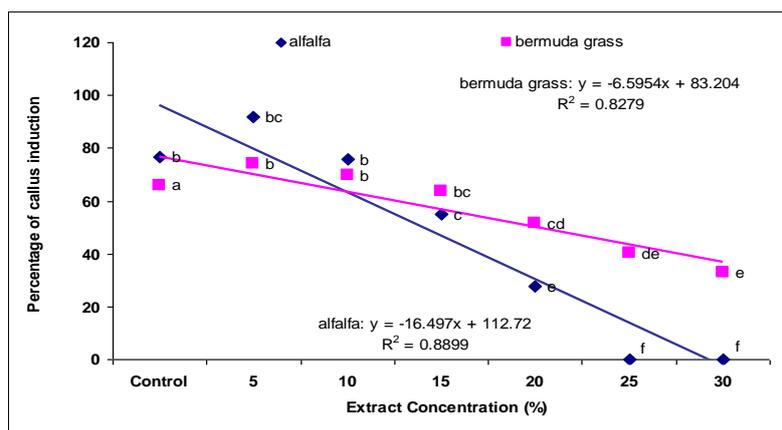


Figure 4. Effects of alfalfa and bermudagrass extract in different concentration in rye percentage of callus induction

**Callus volume:** Callus volume, under the influence of different concentrations of alfalfa and bermudagrass extracts, significantly decreased. The reduction as a result of the treatment using alfalfa extracts with concentrations of 15 and 20 % were 30 and 58 % respectively. The reduction of the volume of callus was lower with *C. dactylon* extract than that with *M. sativa* extract (Figure 3). Allelopathic compounds reduce cell division and cell growth (10). The decrease of callus growth by allelopathic compounds has also been proven in other researches. In a research by Yang and Futsuhara (32), soybean callus and rice callus were placed in a culture medium. The allelopathic effect of rice was so severe that the rate of soybean callus growth decreased 100 times in the experimental conditions. Other studies showed that blocking effects were observed because of the volatile compounds from rice callus. Simple linear regression showed that for every one-unit increase in the concentration of alfalfa *M. sativa* extract caused a decrease of 0.69 units in rye embryo callus, and *C. dactylon* extract caused a decrease of 0.31 units in the of volume callus rye embryo (Figure 5).

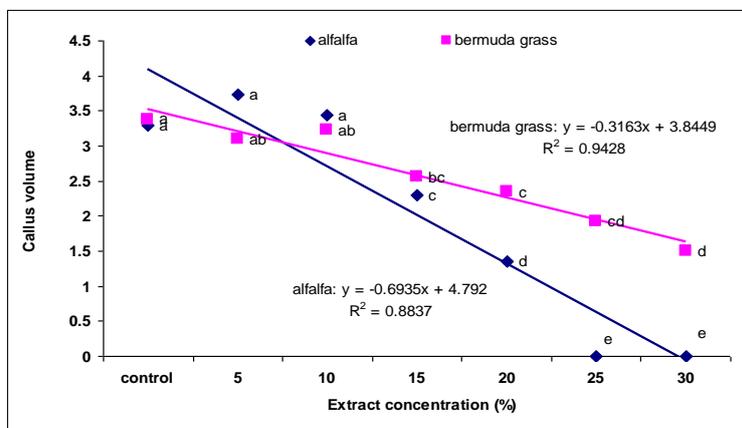


Figure 5. Effects of alfalfa and bermudagrass extract in different concentration in rye callus volume

**Fresh weight of callus:** With increasing amounts and volumes of callus in the treated 5 % extracts of alfalfa, rye callus fresh weight also increased (Figure 6). Abdul-Rahman and Habib (1) reported that Benzoic acid is among allelopathic compounds in alfalfa, that in low concentrations of Benzoic acid and its derivatives, 3, 4 Dihydroxy Benzoic Acid act like auxin. The fresh weight of the callus decreased by 35 and 75 %, respectively, with concentration increases of 15 and 20 %. Increasing the concentration of bermudagrass extract up to more than 5 % caused a significant decrease in the fresh weight of the callus. Extract concentrations of 10, 15, 20, 25 and 30 %, decreased the fresh weight of callus to 34, 31, 54, 68 and 80 %, respectively. Simple linear regression showed that for every one-unit increase in the concentration of alfalfa extract, 0.011 units of rye embryo callus fresh weight decreased, and bermudagrass extract reduced 0.007 units of the fresh weight of rye embryo callus (Figure 6).

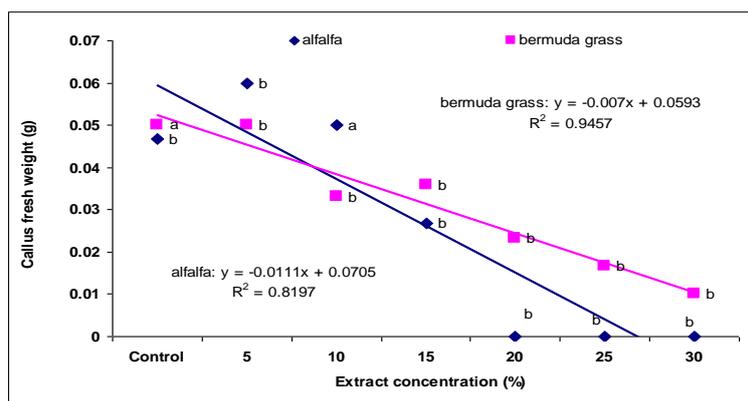


Figure 6. Effects of alfalfa and bermudagrass extract in different concentration in rye callus fresh weight

**Dry weight of callus:** Changes in the dry weight of callus, matching the changes in the volume and fresh weight of callus, was affected by the different concentrations of alfalfa and bermudagrass extracts. Alfalfa extract with a concentration of 5 % caused a 17 % increase in the dry weight of callus compared to control conditions (Figure 7).

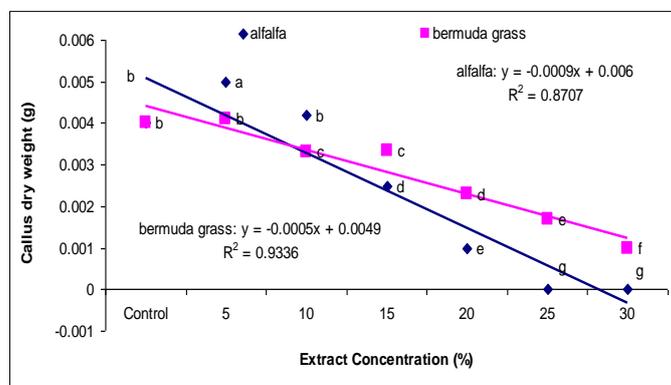


Figure 7. Effects of alfalfa and bermudagrass extract in different concentration in rye callus dry weight

Zahedi and Ansari (34) also reported that chemical compounds that stop the growth of some plant species can have a stimulating effect in other concentrations or in other species. Moreover, 15 and 20 % concentrations of alfalfa extract decreased 50 and 72 % of dry weight of rye callus respectively. Increasing concentration of the extracts caused a significant decrease in the dry weight of callus. Concentrations of 10, 15, 20, 25 and 30 % of extract decreased the dry weight of callus to 20, 20, 45, 60 and 72 % respectively (Figure 4). Increasing the concentration of allelopathic extract will always have negative effects on the physiological processes of the plants (12). In a study by Mucciarelli *et al.* (23), 1000 mmol concentration of 3,4- Dihydroxy Benzoic Acid tremendously reduced callus, shoot and root growth in plants. The results of this study indicated that the inhibitory effects of (*M. sativa*) extracts on rye callus generation was more than (*C. dactylon*) extract. Cell division is the most sensitive stage to allelopathic combination, so allelopathic materials can severely affect this process. This noticeable reduction of cell division can lead to the reduction of callus induction and callus volume. Phenolic compounds possess the power to alter the mitochondrial membrane, which prevents the energy needed for vital cell division and growth responses from being transmitted. This instability results in several physiological changes that retard growth and decrease callus formation.

### RYE REGENERATION

**Rooting (%):** The application of extract with different concentrations of 15, 20 and 25 % decreased the rooting percentage in rye to 40, 63 and 69 %, respectively. The application of extract with 30 % concentration stopped rooting in rye (Figure 8).

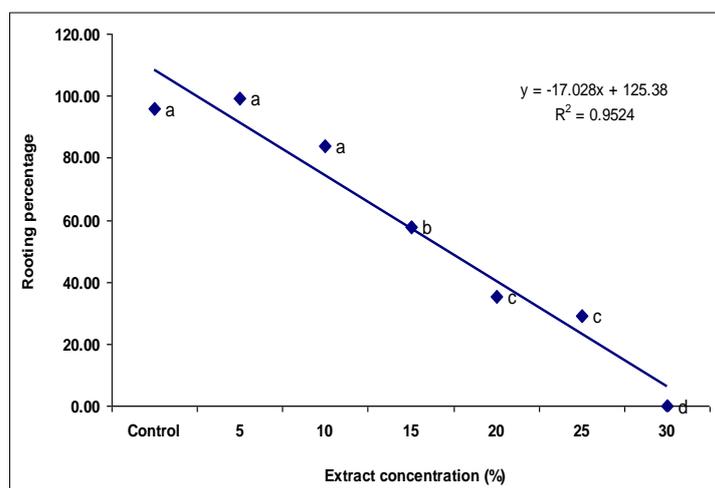


Figure 8. Effects of different concentration extracts in rooting percentage

Mucciarelli *et al.* (23) reported that allelopathic compounds could reduce callus rooting. Linear regression showed that every one-unit increase in extract concentration reduced the production of roots of rye callus up to 17.02 % (Figure 8). Alfalfa (*M. sativa*) extracts had more allelopathic properties than bermudagrass (*C. dactylon*) extract in rooting % (Table 2).

Table 2. Effect of alfalfa and bermudagrass extract in rye regeneration traits.

Extract	Rooting (%)	Root length (cm)	Shooting (%)	Shoot length (cm)	Seedling length (cm)
Alfalfa	55.84312	2.898635	68.32919	2.901672	5.800307
Bermuda grass	58.69382	3.216905	56.29331	2.426238	5.643142

**Root Length:** The study indicated that 10, 15, 20 and 25 % concentrations of extract decreased the percentage of root length to 21, 49, 73 and 79 % respectively. In 30 % concentration, root length was considered to be zero due to the lack of root generation (Figure 6). The cells around the root were contracted as a result of allelopathic substances, and the cell architectures were disorganized with barely noticeable differences between them. Although the nuclei of root tip cells are inactive and disorganized, the presence of vacuoles indicates that some of them are actual cellular components (13). Abenavoli *et al.* (2) reported that allelopathic compounds can reduce root growth. Simple linear regression showed that for every one-unit increase in extract concentration, 1.025 units of root length production in rye callus decreases (Figure 9). Alfalfa (*M. sativa*) extracts had more allelopathic properties than bermudagrass (*C. dactylon*) extract in root length (Table 2).

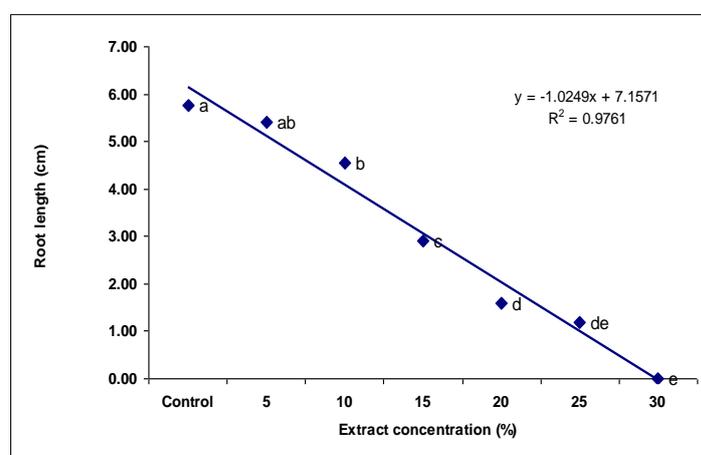


Figure 9. Effects of different concentration extracts in root length

**Shooting (%):** Bermudagrass (*C. dactylon*) extracts had more allelopathic properties than alfalfa (*M. sativa*) extract in shooting percentage (Table 2). The results indicate that there was not a significant difference between control sample and 5 and 10 % extracts in terms of shooting percentage, but increasing the extract concentration reduced the shooting %. Extract concentrations of 15, 20, 25 and 30 % also reduced the shooting percentage 31, 50, 55 and 81 % respectively (Figure 7). Weir *et al.* (31) and Jimenez (14) reported that plant hormones play an important role in the production of embryo from callus. The percentage of bermuda grass extract affected shooting was less than that of alfalfa (*M. sativa*) extract. Simple linear regression showed that for every one-unit increase in extract concentration, 13.39 % per unit of rye shooting percentage would decrease (Figure 10). Therefore, the

production of roots from callus, using allelopathic extract alfalfa (*M. sativa*) and bermudagrass (*C. dactylon*) is more sensitive than the production of shoots from callus.

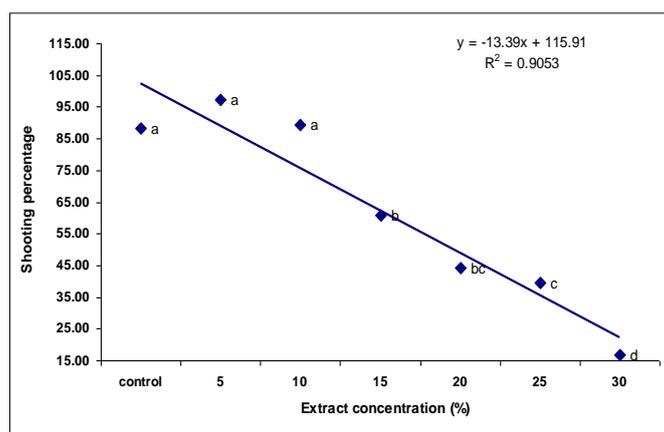


Figure 10. Effects of different concentration extracts in shooting percentage

**Shoot Length:** Bermudagrass extracts had more allelopathic properties than alfalfa (*M. sativa*) extract in shoot length (Table 2). The length of the shoot produced from rye callus, was not significantly affected by 5 and 10 % concentrations of bermudagrass (*C. dactylon*) and alfalfa (*M. sativa*) extract. Extract concentrations of 15, 20, 25 and 30 % reduced the shoot length by 44, 64, 73 and 91 %, respectively (Figure 8). As a result, the allelopathic effects of the extract on the growth and production of the shoots increased with the increase in extract concentration. The pH, osmotic potential, and total amount of allelopathic compounds all increased as allelopathic compound concentrations rose, causing

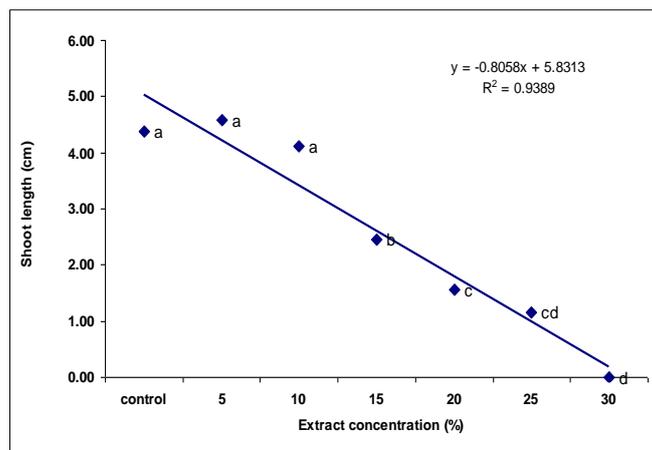


Figure 11. Effects of different concentration extracts in shoot length

them to have more inhibitory effects (28). Bermudagrass (*C. dactylon*) extracts had more allelopathic properties than alfalfa (*M. sativa*) extract in shoot production (Table 2). Simple linear regression showed that for everyone-unit increase in extract concentration, 0.806 units of the length of rye shoot would decrease. Thus, root production is more sensitive to allelopathic extracts of alfalfa (*M. sativa*) and bermudagrass (*C. dactylon*) than shoot production (Figure 11).

**Seedling length:** Bermudagrass (*C. dactylon*) extracts had more allelopathic properties than alfalfa (*M. sativa*) extract in seedling length (Table 2). Seedling length decreased with the increase of the concentrations of extract. Extract concentrations of 10, 15, 20 and 25 % led to a reduction of 15, 47, 69 and 76 % in seedling length in control conditions. In 30 % extract concentration, the seedling length was zero (Figure 12). Allelopathic compounds reduce cell division by reducing the functioning of gibberlin and Indole acid (18). Allelopathic compounds also reduce the growth of cells (7). So, the allelopathic compounds can reduce the length of different parts of seedling by reducing cell division and elongation. Simple linear regression showed that for every one-unit increase in extract concentration, 1.83 units of seedling length produced by rye callus would decrease (Figure 12).

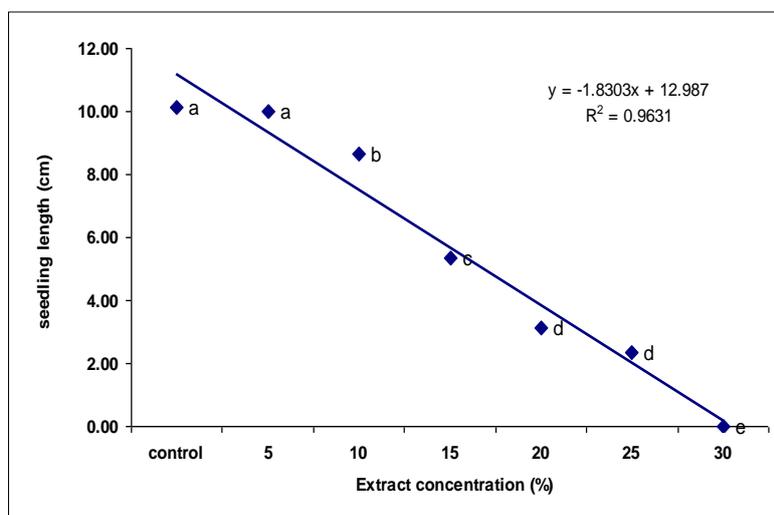


Figure 12. Effects of different concentration extracts in seedling length

Termination or delay of reserved materials activity, which usually occurs fast during rooting and shooting, can reduce respiration reaction and finally continues reduction of ATP in callus exposed to allelo-chemicals. Disorder in respiration leads ATP energy limitation and then decreases shoot and root growth. Flavenoids are such compounds that exist in these plants (alfalfa (*M. sativa* L.) and bermudagrass (*C. dactylon*). They are such important compounds that decrease seedling growth because of avoiding the energy transmission. In addition to reducing calluses, allelopathic actions also delay roots and shooting. Additionally, impaired photosynthesis-related element absorption can affect the growth of roots and shoots. This may occur when allelopathic substances are used in

regeneration. The most significant classes of allelopathic compounds in these plants, such as phenolic acids, inhibit mineral element absorption, transmission from the root to other areas, and root growth. Phenolic acids may also hinder seedling growth. The primary source of seedling development acceleration through carbohydrate generation is photosynthesis.

## CONCLUSIONS

Rye callus induction significantly decreased with increasing concentration of allelopathic extracts of both (*M. sativa* L.) and (*C. dactylon*) and alfalfa extract concentrations of 25 and 30 % prevented callus induction in rye. The reaction of characteristics such as callus volume, fresh and dry weight of the callus to the increasing concentration of the extracts of bermudagrass and alfalfa was similar to their reaction to callus induction. The negative effects of alfalfa extracts on the mentioned attributes were more than those of bermudagrass. Rye embryo and its components were affected by extracts of these two plants, and the rooting of the produced callus in 30 % concentration stopped, but the production of shootings didn't stop despite its 91 % decrease. The alfalfa (*M. sativa* L.) extracts had greater effect on rooting, and bermudagrass (*C. dactylon* L.) extracts had greater effect on shooting.

Nowadays, the global human efforts in agriculture is to reduce the use of pesticides by introducing new ecological methods. The compounds in medicinal and aromatic plants have allelopathic properties to control crops pests including the weeds using non-chemical methods such as allelopathy. Allelopathic studies of medicinal plants can lead to the discovery of natural herbicides and new growth inhibitors. Therefore, the extract used in this research needs to be investigated on a wide range of other plants as well.

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## COMPLIANCE WITH ETHICAL STANDARDS

**Author Contributions:** M.Y., P.B, conceptualization, visualization, review, supervision, analysis, writing-original draft, review and editing; P.B. and F.S.: literature search, collection of data, review and editing. All authors have read and agreed to the published version of the manuscript.

**Data Availability Statement:** Not applicable

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## DECLARATION

Kanchaveli, Sh. S. designed and planned experiment. Chkhubadze G. Conducted experiment in greenhouse and plantation. Shainidze O.T. conducted experiments in the laboratory and wrote article.

## CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

## ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct

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