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Original Article

Genetic enhancement of phytotoxic activity of *Trigonella foenum-graecum* L.

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T. foenum graecum
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Allelopathic potential
Lettuce

Abstract

Allelochemicals are proposed to play a major role in the allelopathy mode of action. However, these compounds are available in low concentration in most allelopathic plants. *Trigonella foenum graecum* L. (fenugreek, Fabaceae family) attracted the attention of many researchers and its allelopathic potential was highlighted in their works. In the present study, we evaluated the effect of colchicine, a polyploidizing substance, on the allelopathic activity of *T. foenum graecum* a diploid plant. Myxoploid plants are obtained by immersion of fenugreek seeds in colchicine solution at 0.5%. Aqueous and organic extracts (petroleum ether, ethyl acetate and methanol) from myxoploid and diploid plantlet shoots were assayed to determine their allelopathic potential by evaluating their effect on lettuce seed germination and seedling development. Results showed that colchicine enhances significantly fenugreek allelopathic capacity. Myxoploid plant extracts had a much more significant effect in all concentrations used than those from diploid plants. Aqueous extracts from diploid plantlets tested at 40g/L induced an inhibition percentage of seed germination of 36.7% against 13.3% in contact with myxoploid plant extracts. At 6000 ppm, methanolic extract obtained from myxoploid plants was the most toxic and was the only to inhibit lettuce seeds germination. Seedling growth was strongly inhibited in the presence of these extract. The concentration giving an inhibition of 50% for root growth (IC_{50}) was equal to 2500 and to 2250 ppm of diploid and myxoploid plant extracts, respectively. For shoot growth, the IC_{50} was equal to 5500 and 2500 ppm in contact of diploid and myxoploid plants, respectively. The use of the colchicine for the allelopathic potential improvement is discussed.

Mots clés :
T. foenum graecum
Amélioration
Colchicine
Potentiel allélopathique
Laitue

Résumé

Amélioration du potentiel génétique de l'activité phytotoxique de *Trigonella foenum-graecum* L. Les allélochimique sont proposés pour jouer un rôle majeur dans le mode d'action du phénomène allélopathique. Cependant, ces composés sont disponibles en faible concentration dans la plupart des plantes allélopathiques. *Trigonella foenum graecum* L. (fenugrec, Fabaceae) a attiré l'attention de plusieurs chercheurs. Son potentiel allélopathique a été mis en évidence. Dans la présente étude, nous avons évalué l'effet de la colchicine, substance polyploïdisante sur ce potentiel. Les plantes myxoploides sont obtenues par immersion des graines de fenugrec dans une solution de colchicine à 0,5%. Les extraits aqueux et les extraits organiques (à l'éther de pétrole, à l'acétate d'éthyle et au méthanol), des parties aériennes des plantes diploides et celles myxoploides ont été testés afin de déterminer leur pouvoir allelopathique et évaluer leur effet sur la germination des graine de fenugrec et sur le développement des plantules. Les résultats ont montré une amélioration significative de la capacité allélopathique de fenugrec traité par la colchicine. Les extraits des plantes myxoploides testés à différentes concentrations ont montré un effet beaucoup plus important. L'extrait aqueux des plantes diploides testé à 40 g/l et au 7ème jour, induit un pourcentage d'inhibition de la germination de 36,7% contre 13,3% en présence d l'extrait des plantes diploïdes. À 6000 ppm, l'extrait méthanolique des plantes myxoploides est le plus toxique, de plus il est le seul qui a provoqué une inhibition totale de la germination des graines de la laitue. La croissance des plantules est dose-dépendante et a été largement inhibée en contact du dernier type d'extrait. La concentration de l'extrait des plantes diploïdes et celle des plantes myxoploides entrainant une inhibition de la croissance de la racine de 50% (IC_{50}) est respectivement de 2500 et 2250 ppm. Pour la croissance des parties aériennes la IC_{50} est respectivement de 5500 et 2500 ppm. L'utilisation de la colchicine pour l'amélioration du potentiel allélopathique est discutée.

INTRODUCTION

At present, there is a trend towards searching for novel natural plant products to develop bio-herbicides and bio-pesticides. Numerous plants are reported to possess allelopathic potential and efforts have been made to apply them for weed or harmful fungi control. Although most common allelopathic plants have potential for weed suppression, their effects are usually short-lived and weeds re-emerge (Xuan et *al*., 2004; El Ayeb et *al*., 2013). Furthermore, to successfully suppress the initial growth of weeds, a large amount of plant material (at least 1–2 $t.ha^{-1}$) needs to be added to the soil. This would require a large labour force. The isolation and identification of allelochemicals in higher plants has been attempted, but the identified allelochemicals were mostly in low concentrations and interactive. So improve plant allelochemicals content is a great challenge.

It is largely registered that polyploidy would improve plant vigour and size. About half of plant species are polyploid (Hirsch, 2001). Among the most known polyploid species, we can quote wheat (hexaploïde), leeks (tetraploid), cutter (octaploïdes), some egg-plant species (tetraploid) (Hirsch, 2001). To these natural polyploids, we can add forms created by man: red beet, turnip, clovers, rye-grass (Bilquez, 1956 ; Hirsch, 2001). Polyploidy induced, inter alias, the multiplication of each gene present in the diploid parental organization witch could influence characters governed by these genes. Accumulation of additive genes by polyploidy has consequences at cellular, physiological, biochemical and morphological levels. Experimental autopolypoids production allowed obtaining beets richer in sugars, tomatoes richer in vitamins, larger fruits, a more abundant fodder mass... (Bilquez, 1956). Mathura *al*., (2006) found that chlorophyll content is increased in tetraploid plants of *Acacia mearnsii*.

Autotétraploids Corn with white grains is less rich in carotene than diploids one, whereas autotétraploids corn with yellow grains are richer in carotene than the diploids (Bilquez, 1956). These works underline the great utility of polyploidy in the improvement programmes of plants with an aim to pass, to certain cultivated species, the economic characters present in some wild species (allopolyploïdisation) or to have plants with higher agronomic qualities (autopolyploïdisation) (Bilquez, 1956 ; Dubois, 1989).

Autopolyploidy should be an effective means to obtain plants of higher quality, hence the majority of crop plants with a great economic interest have a multiple chromosomal number than that of the wild species, and natural autoploids cultivars coming from known diploids forms, have dimensions and robustness higher than those of the diploids. Authors thought benefiting from this observation and tried to produce in their laboratories autoploid plants employing some agents. Results are variable according to the polyploïdising agent nature and the treated biological material. Among these agents, we can quote irradiation (X-ray), electric field, centrifugation of the germinated seeds or cal, nitrogen protoxide (NR_2O), acenaphtene, the α-monoiodonaphtalene, phenyluretane, paradichloro-benzene, cold-heat shock method (Eigsti, 1957). But nowadays, the most used agents remain the inhibiting agents of the spindle mitotic blocking consequently the cellular division like oryzaline and colchicine (Vainola, 2000 ; Blakesly et *al.,* 2002 ; Adanyia and Shirai, 2001 ; Jesus-Gonzalez and Weathers, 2003 ; De Carvalho et *al.*, 2005). Colchicine is water soluble and effective in much diluted solutions at concentration from 0.1 to 0.5% (Eigsti, 1957 ; Buffaloe, 1959 ; Badoc et *al.,* 1998). Colchicine is abundant in nature. It is a toxic white powder obtained from colchic, *Colchicum autumnale* L. It is a tricyclic alkaloid ($C_{22}H_{25}NO_6$). It blocks the cellular division without acting on the DNA, by blocking the microtubules polymerization (Kim et *al.*, 2002 ; Niel and Scherrmann, 2006). This action causes a fast disappearance of the mitotic spindle, avoiding thus the chromosomes polar migration at the anaphase and the proliferation cellular process stop, inducing consequently the formation of cells with a double chromosomes number (Tambong et *al.,* 1998 ; Blakesly et *al.,* 2002). Colchicine importance effect depends on the physiological activity of the embryonic cells at the treatment time. For example, germinated seeds do not give same results as dry ones (Eigsti, 1938). It is known that colchicine can induce high percentages of chimaeras (Wan et *al.,* 1989) considering that it influences only the cellular division and consequently the polyploïdisation does not affect, at equal share all plant cells (De Carvalho et *al.,* 2005) but this does not neglect its tétraploïdisant capacity proven by Gmitter et *al*., (1991). The tetraploids DNA content is doubled compared to the diploids (Blakesley et *al.,* 2002). In the literature, authors studied especially the effect of colchicine on the agronomic characters (germination percentage, cuts flowers, fructification percentage, etc.) and genetics (chromosomal number, nucleolus number, meiotic anomalies, etc). Fenugreek, (*Trigonella foenum graecum* L.) is an annual legume, considered as productive medicinal species of active ingredients and also fodder plant with the economic repercussions. Works concerning the allelopathic potential of *T.* foenum-*graecum* are very few. This species showed an insecticidal activity against *Tibolium castaneum* and *Acanthoscelides obtecus* (Pemong et *al*., 1997). Evident et *al*., (2007) worked on root exudates of fenugreek which showed an inhibiting effect on germination of a bad grass *Orobranche crenata,* parasite of leguminous plants. From these exudates, they isolated allelopathic substance Trioxazonane. The same authors showed as the intercultural *T foenum-graecum* with other leguminous plants (bean, pea, lens and chickpea) reduced their infection by *Orobranche crenata* (Fernandez-Aparicio et *al*., 2007). More recently, the allelopathic potential of the aqueous extract of various plant parts of fenugreek was evaluated on a series of seed crops and the authors recorded a greater toxicity of shoots with a selective effect (Haouala et *al*., 2008).

The aim of this work is to evaluate the effects of colchicine treatment on the allelopathic potential of fenugreek on crop species and bad grasses through bioassay and glasshouse. This research will promote a method for enhancing allelopathic potential of plants via improving of the genetic potentialities of *Trigonella foenum-graecum*.

MATERIALS AND METHODS

Plant materials

Fenugreek seeds were surface sterilized immersing 0.525 $g.L^{-1}$ sodium hypochlorite for 15 min. The seeds were rinsed four times with deionised water, carefully blotted with paper towel and immediately sown in Petri–plate. After 24h, germinated seeds were divided into two sets (i): first set is let continue to germinate and grow, to obtain diploid plants ii) the second germinated seeds set is immersed in a colchicine solution at 0.5% during 4h then abundantly rinsed with the distilled water. They were replaced in Petri dishes to continue germination, to obtain myxoploid plants. Germination was carried out in Petri dishes with Whatman No.1 filter paper soaked with distilled water. The one week old seedlings are planted in pots [40 cm length, 30 cm broad and 20 cm dept] and placed under the natural conditions of illumination during March-April 2007. The plants are irrigated daily with water of tap. Shoots of diploid and myxoploid plants were collected. After cleaning with tap water, the fresh material was oven-dried at 60 °C for 2 days then was ground into a powder using a Wiley mill and stored at 2 °C until use.

Preparation of aqueous and organic extracts

The treatments consisted of three factors: Aqueous extracts: 8 [10, 20, 30 and 40 g/l for diploid and myxoploid plants], organic extracts: 14 [petroleum ether, ethyl acetate and methanol at 6000 ppm and dilutions going from 6000 to 2000 ppm for methanolic extract for diploid and myxoploid plants] and one test crops (*Lactuca sativa* L.). Lettuce has been used as tested plant because it is too sensitive to chemicals at low concentration, as it sometimes over-estimates the actual allelopathy (Olofs-dotter, 2001).

Forty grams of dried shoots of fenugreek diploid and myxoploid plants were separately extracted by soaking in 1L distilled water at 24°C for 24h in shaker to give a concentration of 40g dry tissue L^{-1}. The extract was filtered through two layers of cheesecloth to remove the fibre debris, and centrifuged at 4530 g for 2h. The supernatant was vacuum filtered again through Watman No.42 filter paper (Chon et *al*., 2003).

The powdered material of shoots of fenugreek diploid and myxoploid plants (75g) was successively extracted with petroleum ether during 72h at ambient temperature and then filtered using Watman No.1 filter paper. The filter paper did not contain inhibitory components, because maximum seed germination was recorded when each species was essayed using deionised water. The residue was subsequently extracted with ethyl acetate, followed by methanol to generate two more fractions (Jefferson and Pennacchio, 2003). The fractions were taken to dryness on a rotary evaporator at 40-50°C and transferred in vacuum freeze dryer to obtain dry matters witch were stored at 4°C until use. Yields were calculated (Table 1).

Effect of aqueous diploid and myxoploid plants extracts on lettuce seed germination

Each stock aqueous extract was diluted appropriately with sterile distilled water to give final concentrations of 10, 20, 30 and 40g.L^{-1}. Five millilitres of the extracts was pipetted onto Wathman No.1 filter paper in a Petri dish. Distilled water was the control. Lettuce seeds were surface sterilized immersing 0.525 g.L^{-1} sodium hypochlorite for 15 min. Seeds were rinsed four times with deionised water, imbibed in deionised water at 22°C for 12h, and carefully blotted using folder paper towel. Thirty swollen seeds were evenly placed on filter paper wetted with extract in each Petri dish and kept in a growth chamber [24°C, 14-h light 400 µmol photons.m^{-2}s^{-1} photosynthetically active radiation (PAR), 10-h dark and 22°C]. Three replicates were incubated in a randomized complete block design. The germination was recorded at 24-h intervals till 144 h. A seed was considered germinated when the radical protruded ≥2mm (Hou and Romo, 1998). Data were transformed to percent of control for further analysis. At 7th day after sowing, shoot and root length and dry weight of recipient specie seedlings were measured. Data were transformed to percent of control for analysis. Extract concentration resulting in 50% inhibition of germination and root/shoot length (IR_{50}) of controls were determined by interpolation.

Phytotoxic effects of 3 solvent fractions

The three dried samples concentrated from petroleum ether, EtOAc and MeOH were again dissolved in MeOH to compare their phytotoxic effects. Five millilitres of each of these fraction solutions at 6000ppm and the methanol-only solution (control) were placed in a Petri dish lined with one Watman No.1 filter paper and evaporated to dryness for 24h at 24°C. Methanol extract was assayed at different concentrations: 2000, 3000, 4000, 5000 and 6000ppm, since it is the most toxic solvent. Distilled water was the control. After evaporation, 5 ml of distilled water was piptted onto the filter paper and then thirty imbibed lettuce seeds were placed on the paper and grown for 7 days. Bioassay procedures, conditions and parameter measured were same to the previous work.

Statistical analysis

The laboratory bioassays were conducted with three replications. Duncan-tests and ANOVA were performed on SPSS 13.0, for Windows program, to analyze treatment differences. The means were separated on the basis of least significant differences at the 0.05 probability level.

RESULTS

Yields of organic extracts

Results show that colchicine treatment increased significantly the yields (Table1). Indeed, yields of the myxoploid plant extracts doubled, on average, compared to those of the diploid one.

Table 1: Yields of crud extracts from shoots of diploid and myxoploid plants of *T.foenum-graecum.*

	Diploid	Myxoploid
Petroleum ether	0.8428	1.9121
Ethyl acetate	1.4070	2.5129
Methanol	1.7409	3.0370

Effect of aqueous extracts on seed germination and growth of lettuce

Figure 1 presents the germination kinetics of lettuce seeds in presence of aqueous extracts diploid and myxoploid plants of fenugreek shoots at various concentrations. Results showed that the inhibition of lettuce seeds germination in presence of extracts was dose-dependent (Figure 1). The myxoploid plants extract had a much more significant ($p<0.05$) effect in all concentrations used (Figure 1b). With 40g/L and at 7th day, inhibition percentage was 36.7% and 13.3% in presence of diploid and myxoploid plant extracts respectively (Figure 1). Shoot extracts of fenugreek significantly delayed seed germination with increasing of extracts concentration. This delay was more important with myxoploid plant extract. Both extracts were significant allelopathic inhibitors on lettuce root and shoot length, but the toxicity is higher with fenugreek myxploid plants extracts.

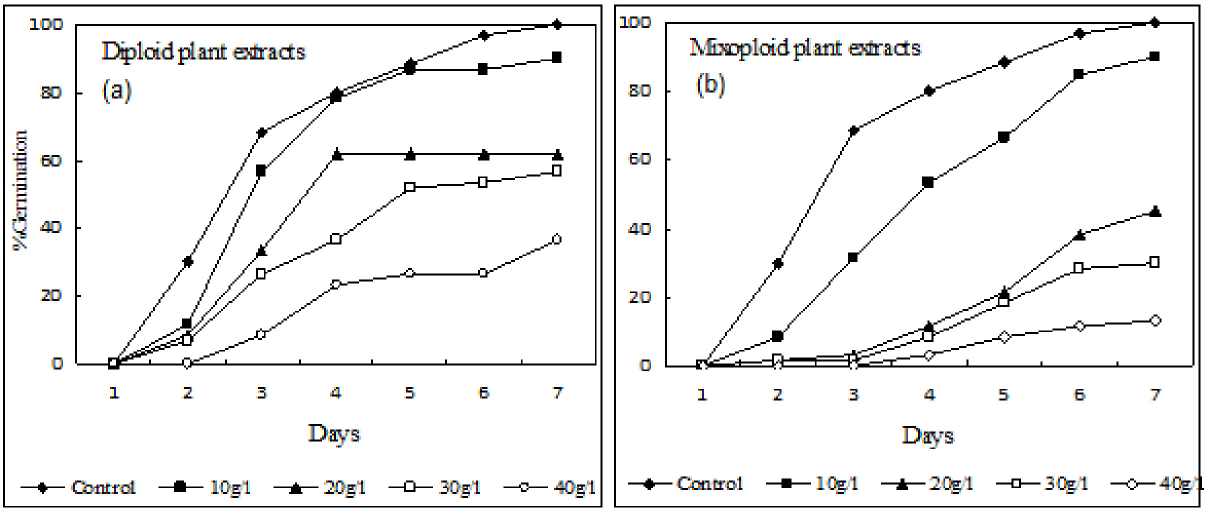

Figure 1. Kinetic germination of lettuce seeds in presence of aqueous extracts of fenugreek shoots of the diploid and myxoploid plants at various concentrations. Distilled water is the control. The bars show standard error.

Root growth is strongly inhibited in presence of aqueous extracts and inhibition is more significant that the extracts were more concentrated and yet more significant when the extract comes from the myxoploid plants (Figure 2). Thus, with 40g/L and at 7th day, lettuce root length is, respectively, 10% and 1% of the control in presence of the extracts of diploid and myxoploid plants (Figure 2a).

In the same way, shoot growth of lettuce was inhibited more and more than the extracts are concentrated. Therefore the extracts of myxoploid plants are much more toxic. With the strongest concentration, the length of shoots was 18% and 0.8% of the control, respectively, in presence of the extracts of diploid and myxoploid plants (Figure 2b). On the other hand, the inhibitory effect on root length is higher than shoot length ($p<0.05$).

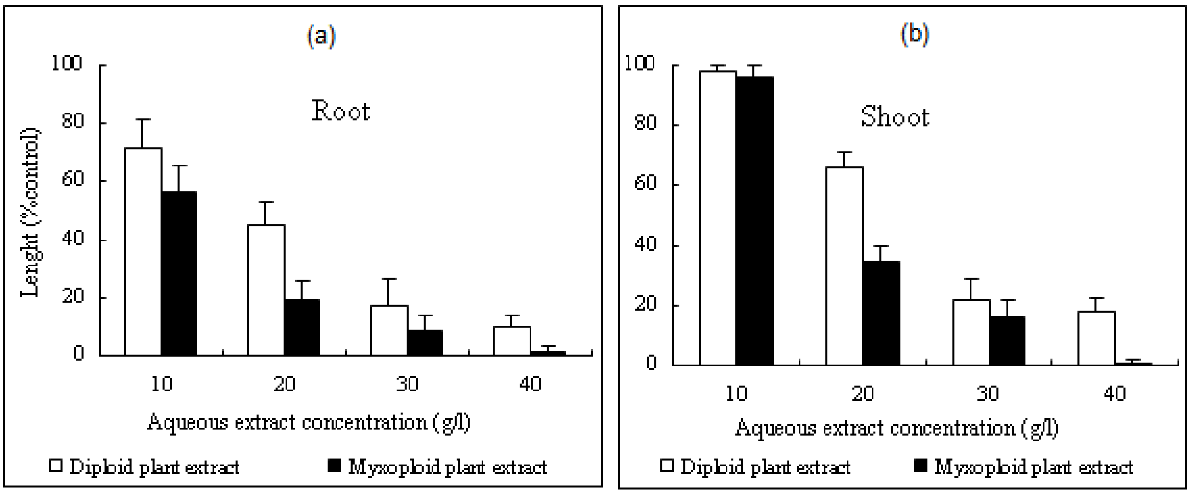

Figure 2. Effects of aqueous extracts of diploid and myxoploid shoots of *T. foenum-graecum* on early seedling growth of roots and shoots of lettuce, 7 days after germination, expressed in % of control.
The bars on each column show standard error.

Effect of organic extracts on seed germination and growth of lettuce

Bioassays in presence of organic extracts showed that methanolic extracts were the most toxic ones. Control bioassay, in the presence of methanol indicated that this organic solvent did not have any significant effect on germination of lettuce seeds. We recorded a delay of 3 days, 4 days and more than 7 days, to reach to 50% of seed germination, in the presence of respectively, petroleum ether, ethyl acetate and methanol extract of diploid plants compared to the control. This delay was of 4, and more than 7 days in the presence of the same extracts of myxoploid plants (Figure 3). At the seventh day, germination percentage was 92%, 65% and 27% of the control in presence of petroleum ether, ethyl acetate and methanol extract, respectively. Myxoploidy increased the toxicity of an average of 28%. Thus, germination percentages were 58%, 39% and 0% of the control in presence of the same extracts of myxoploid plants (Figure 3).

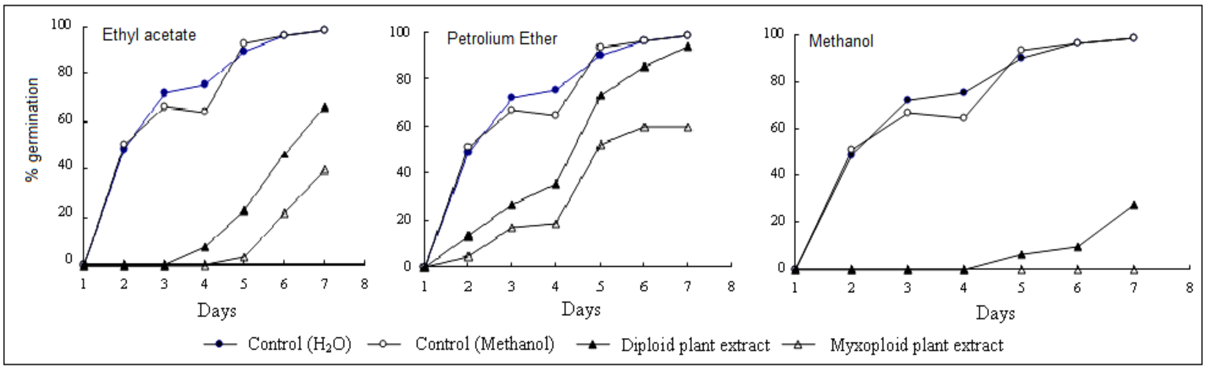

Figure 3. Effects of organic extracts (ethyl acetate, petroleum ether, methanol) of diploid and myxoploid shoots of *T. foenum-graecum* on germination of lettuce seeds expressed in percent. The bars on each column show standard error.

For seedling lettuce growth, we recorded a strong toxicity of methanolic extract followed by ethyl acetate then petroleum ether one (Figure 4). This toxicity was increased by an average of 18% by myxoploidy. Indeed, we registered a total inhibition of root and shoot growth in presence of methanolic extract of myxoploid plants (Figure 4). In presence of ethyl acetate extract of the same plants, the two organs growth was only 5% of the control, whereas it was 23% and 12% of the control for root and shoot, respectively, in presence of the same extract of the diploid plants (Figure 4).

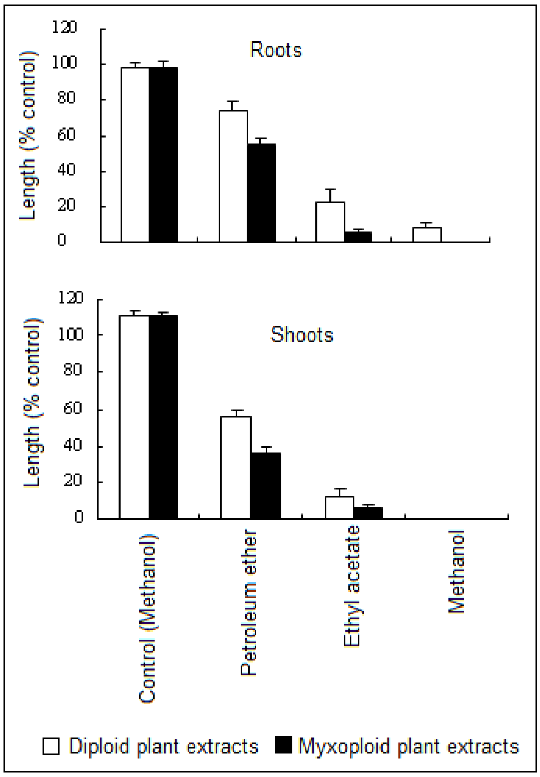

Figure 4. Effects of organic extracts (ethyl acetate, petroleum ether, methanol) of diploid and myxoploid shoots of *T. foenum-graecum* on early seedling growth of roots and shoots of lettuce, 7 days after germination, expressed in % of control. The bars on each column show standard error.

Phytotoxicity of methanolic extract

Methanol extracts which showed most inhibition to aqueous and the others organics extracts, were essayed against lettuce with different concentration (Figure 5). Their effect varied with concentration. The IC_{50}, concentration corresponding to an inhibition germination of 50%, is of 5500ppm in presence of diploid plants extracts and of 4000ppm in presence of myxoploid plants extract (Figure 5). Delay germination is also more important with myxoploid plant extract. With 6000ppm the percentage of germination was only 27.77% with diploid plant extract (Figure 5). Myxoploidy increased strongly methanolic extract toxicity, since with 6000ppm we note a total absence of the germination (Figure 5).

Many works reported that some allelochemicals delay germination (Shariati, 2005).

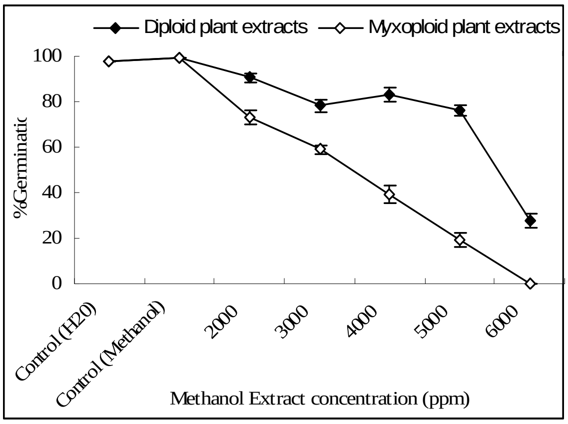

Figure 5. Effects of methanol extract concentration of diploid and myxoploid shoots of *T. foenum-graecum* on germination of lettuce seeds expressed in percent. The bars on each column show standard error.

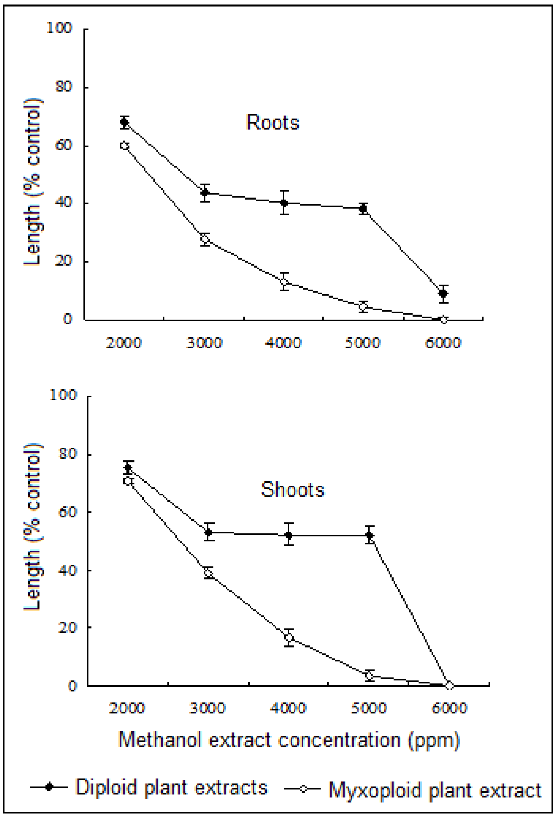

Figure 6. Effects of methanolic extract concentration of diploid and myxoploid shoots of *T. foenum-graecum* on early seedling growth of roots and shoots of lettuce, 7 days after germination, expressed in % of control. The bars on each column show standard error.

Experiments done on several plant species indicate that ABA plays a key role in induction and maintenance of dormancy. Thus change in dormancy may be related to changes at the level of allelochemicals and ABA probably induced by increase of chromosomes charge of cells by colchicines.

DISCUSSION

Studies on enhancement of chemical components are few. The colchicine application on *Matricario chamomilla,* (Asteraceae), led obtaining tetraploid plants with a strong content of sesquiterpenes, particularly chamazulene and α-bisabolol (Badoc et *al.,* 1998). However, this alkaloid did not induce significant variation of the content or the composition of the fruits Fennel essential oil (*Foeniculum vulgare* Mill.) (Badoc et *al.* 1998).

Inhibition germination by aqueous extract is reported in many works (Bulut et *al*., 2006 ; Sarkar et *al*., 2012 ; El ayeb et *al*., 2013), and the degree of inhibition was largely dependent on the concentration of the extracts. Our findings are consistent with those reported elsewhere for other species in a variety of plant families (Escudero et *al*., 2000 ; Macias et *al*., 2000 ; Jefferson and Pennacchio, 2003). Delayed seed germination by an allelopathic extract is due mainly to toxic factors of the leaf extract (Chon *et al*., 2004). It is well reported that aqueous extracts of some plants inhibit seedling growth (Lydon et *al*. 1997 ; El ayeb et *al*., 2013) root and shoot growth (Athanassova, 1996 ; El ayeb et *al*., 2013) germination and induce mortality of plants (Eyini et *al*., 1996).

Relative toxicity of organic extracts is reported in literature. Chon et *al*. (2005) unregistered a higher phytotoxicity of methanol extracts from hexane and EtOAc fractions of lettuce compared to those of BuOH and water fractions. The result suggests that phytotoxic substances were more present in the methanol and EtOAc fractions than in the petroleum ether resulting in more inhibitory effects on the test plant. Inhibition of germination and early growth by organic extract are reported in literature. Bulut et *al*., (2006) registered 77 and 81% of the control for *Pelargonium* seed germination in presence of ethyl acetate and petroleum ether extracts, respectively. The highly significant toxicity of methanolic extract compared with the two other extracts, indicates that the responsible agents for the allelopathic effects of fenugreek are soluble in methanol. They may be phenolic compounds, which are well known potential phytotoxins (Seal et *al*., 2004). Although mathanolic extract yield was higher from myxoploid plant and it showed higher inhibition in germination percentage and seedling growth compared to that from diploid one. This may be due to differences in quality and quantity of allelopathic compounds that were isolated. Inhibition germination would be attributed to allelochemicals like terpinen 4-ol witch completely inhibit *Pelargonium* seed germination at 1000 and 5000ppm (Bulut et *al*., 2006), by inhibiting the cell division in the embryonic mersitems of the seeds (Agbagwa et *al*., 2003). Plant phenolics play a major role in allelopathy (inderjit, 1996). Aqueous extracts of shoot fenugreek exerted a typical effect on germination and seedling growth of lettuce, severe toxicity at high extract concentration and moderate toxicity at low concentration. Lettuce roots appeared to be more sensitive to fenugreek extracts than shoots, presumably because the roots are in direct contact with the extracts, similar results were reported with other crops by Tefera (2002), Bnomik and Doll (1984) and Qasem (1995).

Dose-dependent response of germination was recorded by Bulut et *al*. (2006), who registered 56.67 and 63.33%, 60 and 70% and 80 and 56.67% with ethyl acetate, petroleum ether and chloroform at 1000 and 5000ppm, respectively.

There are no studies related on enhancement of allelopathy potential of plants. Allelochemicals are synthesized in plants as secondary metabolites and located in certain specialized organs of donor plants (Kobayashi, 2004). Viles and Reeses (1996) showed that the chemical activity of specific extracts of *Echinacea angustifolia* was not uniform, but dependent upon genotypic variation of the plants grown in a common garden.

CONCLUSION

The colchicine treatment of *Trigonella foenum-graecum* involved a significant enhancement of the allelopathic capacity of this plant. Indeed, the reduction in the percentage of germination was more significant with the increase in the concentration of the aqueous extract of the myxoploïd plants compared with that of diploid one. In the same way, seedling growth was strongly inhibited in the presence of these extracts. With 6000ppm, organic extracts caused a delay, a reduction in the germination percentage and a reduction growth more or less significant according to solvent. Only methanolic extract of the myxoploid plants showed a total inhibition of lettuce seeds germination. These results indicate that the molecules responsible for the fenugreek allelopathic potential are polar, and that the myxoploidy increased their concentration/effect. To purify, to identify and to proportion these molecules in the two types of plants would be the subject of a later study.

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