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Original Article

Combination Effect of *Piriformospora indica,* Chilling and Gibberellic Acid on Seed Germination Traits of *Kelussia odoratissima* Mozaff.

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Abstract

Kelussia odoratissima Mozaff. belonging to the Apiaceae family, is well known for its medicinal and nutritional importance, endemic to Iran. Seed dormancy is a major problem present in *Kelussia odoratissima* leading to low germination percentage; thus, improvement of seed germination and breaking seed dormancy is important. *Piriformospora indica*, a root-colonizing endophytic fungus, promotes plant growth, development and resistance to biotic and abiotic stresses. In order to evaluate the effects of different treatments of *P. indica* on seed germination traits of *Kelussia odoratissima*, an experiment was conducted based on completely randomized design with five treatments and three replications. The experimental treatments were application of fungal mycelium of *P. indica* (M), spore suspension of *P. indica* (S), the combination of Gibberellic acid and fungal mycelium (H+M), the combination of Gibberellic acid and spore suspension (H+S), and control (C). Based on the results the highest percentage of germination (75%) and the highest germination rate was related to spore suspension of *P. indica*. The lowest amount of germination uniformity (GU) observed in spore suspension of *P. indica* treatment. Among the different treatments, application of *P. indica* spore suspension resulted in lowest times for 10% germination (D_{10}) and 90% germination (D_{90}). The highest plumule and radicle length observed in spore treatment and in comparison with control, application of spore suspension of *P. indica* increased plumule and radicle length by 16% and 32%, respectively. Based on the current results, it seems that spore suspension of *P. indica* was the best treatment for improvement of seed germination traits.

Keywords: Germination uniformity, Medicinal plants, *P. indica*, Seed dormancy

Introduction

Kelussia odoratissima Mozaff (Apiaceae family), locally called "karafs-koohi", is a wild rebus, erect, glabrous, perennial aromatic and medicinal plant. The yellow flowers are 1-2 mm in diameter, all hermaphrodites. It is native to the central region of the Zagros Mountains, Iran, and has a great biological diversity. The temperature in the native habitat of *Kelussia odoratissima* is usually lower than 20 ˚C and includes about120 days of frost, with the temperature reaching below zero in the autumn and winter. *Kelussia odoratissima* Mozaff. is a sweet-smelling plant which has anti-inflammatory, sedative, and antitussive properties [1]. In Iran, this plant is traditionally consumed as a medicinal plant to treat hypertension, inflammation, ulcers, and cardiovascular diseases [2]. Results of previous study showed that the essential oil from the aerial part of the plant contains 23 kinds of different valuable components, of which the major compound is Z-ligustilid [3]. Moreover, some researchers reported an antioxidant property of *K. odoratissima* [2]. In natural conditions, the plant is propagated through seeds that are in general produced once a year in late summer [4].

Because of dormancy problems seeds of *K. odoratissima* often germinate poorly in the nursery, and therefore possibility of propagation of this

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specie through seed is very poor. Seed dormancy could be considered simply as a block to the completion of germination of an intact viable seed under favorable conditions, but earlier reviews concluded that it is one of the least understood phenomena in the field of seed biology [5]. Seed germination could influenced by internal factors of dormancy controlling, including phytohormones (e.g. abscisic acid), and by seed coat factors (seed coat-enhanced dormancy) [6,7]. Dry seeds of most of temperate trees and shrubs, even though mature, will not germinate and grow until they been imbibed to threshold moisture content under cold conditions (0-5 ˚C) (cold stratification) [7,8]. The dormancy of dormant seeds must be broken to induce germination. Various methods are used for this purpose, depending on the plant species and type of dormancy. Chilling treatment plays an important role in providing the required stimulant to overcome dormancy, increase germination, and produce normal seedlings in *Prunus persica* [9], strawberry [10] and wild cherry (*Prunus avium*) [11].

Application of exogenous growth regulator like as gibberellins and cytokinins have been reported as an effective method for breaking seed dormancy and germination improvement in many species [10,12].

Piriformospora indica, a root-colonizing endophytic fungus of Sebacinales, was originally isolated from bush rhizosphere zones of the Thar Desert in India, promoting host plant growth and increasing plant tolerance to biotic and abiotic stresses by affecting physiological properties [13].

P. indica after entering the root cortex, forms inter- and intracellular hyphae. This fungus also forms chlamydospores within or between the cortical cells and hyphae multiply within the host cortical tissues and do not invade the aerial portion of the plant as well. The endophyte *P. indica* like the arbuscular mycorrhizal fungi possesses positive influence on different physiological process of many plant species [14]. *P. indica* enhance the resistance of colonized plants against fungal pathogens and increase the plant tolerance to abiotic stress. Because of ability of this fungus to change the secondary metabolites of different plant *P. indica* can be more economic and beneficial [15].

The exploitation of *P. indica* may be facilitated in future by its ability to grow on axenic artificial solid or liquid media.Vadassery *et al* (2008) revealed that *P. indica* hyphae grown in liquid medium produce free IAA and relatively high levels of cytokinins [16]. Cosme *et al* (2016) showed that *P. indica* helps plants to tolerate root herbivory through changes in gibberellin and jasmonate signaling [17,18]. Also, Khalid *et al* (2018) reported that *P. indica* can affect phytohormone content in *Brassica campestris* ssp. *Chinensis*[19].The main aim of current study was evaluation the effects of different treatments of *P. indica* on seed germination traits of *Kelussia odoratissima*.

Material and Methods

Seed Collection

Mature seeds of *Kelussia odoratissima* Mozaff. were collected in August 2016 from the nature habitat of this plant in Shahrekord, Iran (latitude 32˚ 17´ N longitude 50˚ 51´ E, elevation 2500 m above sea level) and germination experiment started in October 2016.

Fungus Culture

P. indica was cultivated on complex medium [20] for 4 weeks in the dark at 25 ˚C. The spore suspension collected after 28 days by gently scratching the fungus surface on the Petri dishes with a spatula until the spores were released. The spore suspension was then filtered through cheesecloth to remove the excess medium and washed several times with distilled water (dH_2O) containing 0.05% Tween-20. After each washing step, the spores were collected by centrifugation at 4000g for 7-8 min. The spore pellet was finally suspended again in dH_2O and adjusted to 5×10^5 spores per mL. The spore number was calculated using a light microscope and a hemocytometer [20].

For the fungal mycelia process, 8 mm agar discs (fully-grown fungus) were inoculated in 250 ml Erlenmeyer flasks containing 100 ml complex medium without agar on an incubator shaker at 200 rpm at 30±1 °C. After growth for 8 days fungal mycelia were harvested from the liquid culture through filtration and washed with excess of double distilled water to remove adhered salts and the other medium components [21]. This fungal biomass was utilized as second fungal treatment.

Germination Experiment

Uniform sized *Kelussia* Mozaff. seeds were selected for the germination test. The seeds were surface-sterilized in 1% aqueous solution of sodium hypochlorite (NaClO) for 2 minutes and then rinsed with distilled water three times. In order to ensure the viability of the seeds Tetrazolium tet was carried out. Before the main experiment a primary germination test was conducted in room temperature (min 18 °C) but seed germination in this condition totally stopped, therefore the main experiment was done in refrigerator temperature.

Seeds of all treatments were placed on filter paper moistened in Petri dishes in the dark conditions. For spore suspension treatment, first, sterilized seeds were mixed with spore suspension and placed on a shaker for one hour in order to allow the binding of spores to the seed surface. Then the seeds were stored with spore suspension in a refrigerator at 4° C. In fungal mycelium treatment, 1000 mg of mycelium mixed with seeds and stored at 4 ° C. Also in the combination of Gibberellic acid and spore suspension treatment and combination of Gibberellic acid and fungal mycelium treatment, sterilized seeds were mixed with spore suspension and fungal mycelium then they added a solution of Gibberellic acid and stored at 4° C. In control treatment, sterilized seeds directly transferred to Petri dish and stored at 4° C.

The first seeds germinated 38 days after the start of experiment. The number of germinated seeds (>2mm radicle length) from each petri dish was counted every 24 hours. Germination percentage and rate, uniformity and time to 10 and 90 percentage of final germination was calculated using the Germin program [22].

At the end of the experiment, radicle, plumel and seedling length (mm), were measured. The germination rate was calculated according to Ellis and Roberts [23]. The vigor index (VI) was calculated according to Abdul-Baki and Anderson (1973) using the equation 1 [24]:

VI=[seedling length (mm)×germination percentage]. Equation 1

Statistical Analysis

All data were analyzed statistically using SAS software. Mean comparisons were done by least significant difference test (LSD) at 1% probability and the figures were created with Microsoft Excel 2013.

Result

Germination Rate

Results of analysis variance showed that there was significant difference between experimental treatments for germination rate (Table 1). Spore treatment has positive effect compared to control. The highest rate of germination was observed for treatments of spore (0.36 Seed/day), which increased more than 14% compared to control treatment (Fig. 1).

Germination Percentage

The results of analysis of variance for final germination percentage showed that there was a highly significant difference between the treatments (Table 1). The highest percentage of seed germination observed in spore suspension treatment. Compared to the control treatment, the use of spore suspension of *P. indica* increased the final germination by 17% (Fig. 2).

Time to 10% and 90% Germination (D_{10} & D_{90})

There was a highly significant difference for the time to reaching 10% germination (D_{10}) at 1% probability level between treatments (Table 1). The minimum time to reach 10% germination was 25.6 days in spore suspension treatments. This treatment reduced the time to reaching 10% germination (D_{10}) about 34% compared to control (Fig. 3).

Table 1 Analysis of variance (mean of squares) for the effect of experimental treatments on the studied traits of *Kelussia odoratissima* Mozaff. under controled conditions

Source of Variation	df	Germination Rate	Germination Percentage	D 10	D 90	Germination Uniformity	Plumule Length	Radicle Length	Vigor Index
Treatment	4	0.0022 *	188.9 **	314.7**	35.09**	186.9 **	42.6 *	286.8 *	332.3**
Error	10	0.00046	3.32	1.20	5.42	4.29	10.1	55	34.7
CV%		12.9	11.6	8.43	5.66	13.4	16.3	14.8	14.1

The same result also observed for D_{90}. Although spore suspension treatment was more effective than other treatments and the lowest value of D_{90} (61.6 days) observed in this treatment, but difference of spore suspension treatment and control was not significant (Fig. 4).

Germination Uniformity

Result of germination uniformity showed that there was highly significant difference between treatments at 1% probability level (Table 1). The lowest value was related to mycelium treatment with 15.7 days, which reduced more than 23% compared to control. The lower values of germination uniformity means that germination occurred in shorter time and therefore emerged seedlings are more similar.

Plumule and Radicle Length

Results of experiment showed that effect of experimental treatments on plumule and radicle length were significant (Table 1). The highest plumule length (12.8 mm) was related to spore suspension treatment but the difference of this treatment with control was not significant. The lowest plumule length (3 mm) observed in mycelium+GA3 hormone treatment (M+H). The same results also observed for radicle length. The highest radicle length (37.6 mm) observed in spore treatment followed by control (28.5 mm). Radicle length in remaining treatments was lower than control and the lowest radicle length (11.9 mm) measured in M+H treatment.

Vigor Index

Based on results of current experiment the vigor index of *Kelussia* seedling significantly affected by experimental treatments (Table 1). The highest value of vigor index (37.7) was related to spore suspension treatment whereas as a results of application of other treatments, seedling vigor index decreased compared with control.

Discussion

Moist chilling as an effective method resulted to breaking physiological dormancy in the seeds of *Kelussia* and without moist chilling treatment seed germination stopped completely. Application of spore suspension of *P.indica* simultaneous with moist chilling treatment has positive effects on seed germination traits, but under the same condition mycelium treatment resulted to decrease germination percentage.

While the effects of arbuscular mycorrhiza fungi (AM) on plants are relatively well established in adult plants, few studies have investigated whether AM fungi promote seed germination [25,26,27]. Results of previous study on radish (*Brassica rapa* L.) showed that inoculation with arbuscular mycorrhiza fungi significantly affected the final germination percentage and germination rate, but the positive effect of fungus on seed germination rate was higher in comparison with final germination percentage [28].

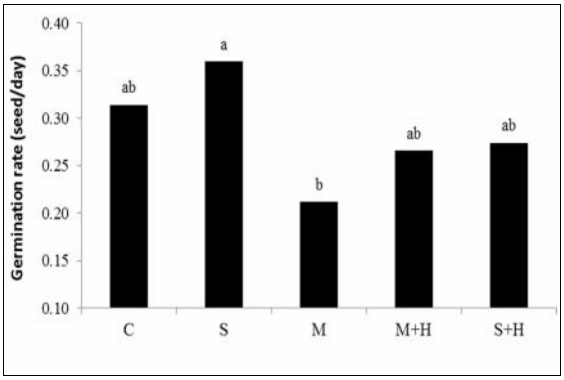

Fig. 1 Effect of different treatments on germination rate of *Kelussia odoratissima* Mozaff.

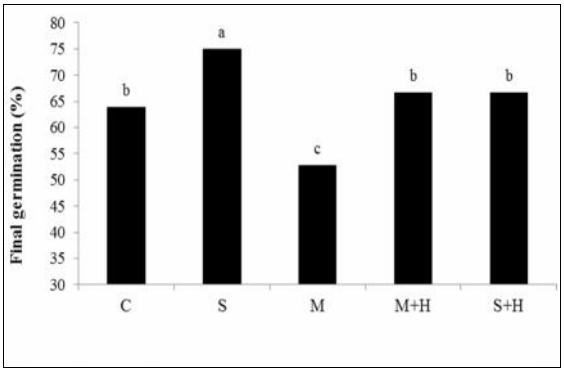

Fig. 2 Effect of different treatments on final germination percentage of *Kelussia odoratissima* Mozaff.

At the seedling stage, the presence of AM fungi has been reported to benefit plant early establishment [29,30]. *P. indica* can significantly mediate improvements in the growth and yield of various crop plants, horticultural and medicinal plants [16,31-38]. The role of *P. indica* inoculation/colonization in medicinal plants has been considered of utmost significance [39]. *P. indica* induced seed germination and development have been reported in several crop plants.

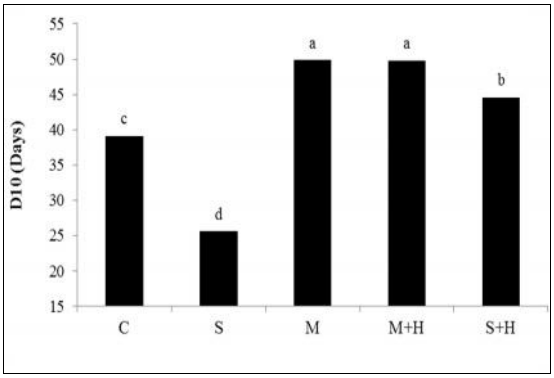

Fig. 3 The effect of different treatments on the time to reaching 10% of germination of *Kelussia odoratissima* Mozaff.

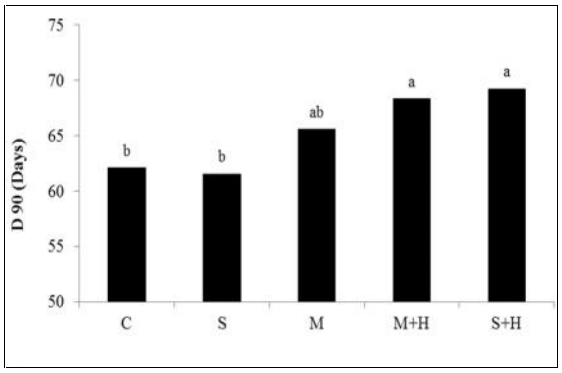

Fig. 4 The effect of different treatments on the time to reaching 90% of germination of *Kelussia odoratissima* Mozaff.

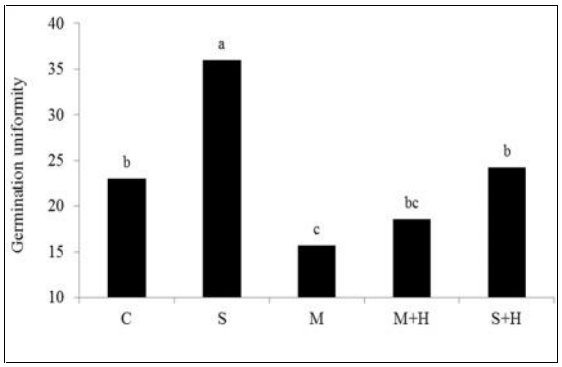

Fig. 5 Effect of different treatments on germination uniformity of *Kelussia odoratissima* Mozaff.

Inoculation of *Oryza sativa* L., *Zea mays* L., *Nicotiana tabacum* L., *Arabidopsis thaliana* (L.) Heynh. and *Brassica oleracea* L. plants with *P. indica* have been shown to have improved seed germination and an increase in seed formation [40]. *P. indica* mediated seed development and enhanced seed production in *A. thaliana* were reported [41]. *P. indica*-inoculated *H. vulgare* seeds exhibited higher viability [42]. Moreover, germinated seedlings immersed in *P. indica*-homogenate exhibited a good survival rate under adverse conditions [42].

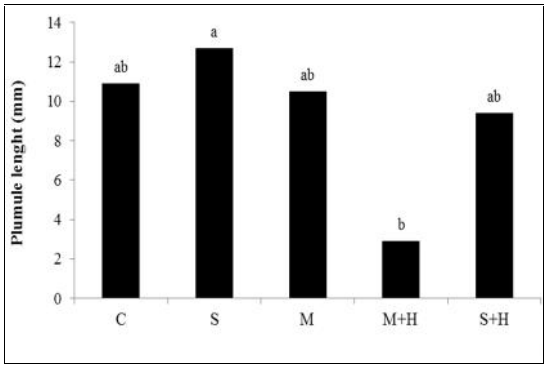

Fig. 6 Effect of different treatments on plumule length of *Kelussia odoratissima* Mozaff.

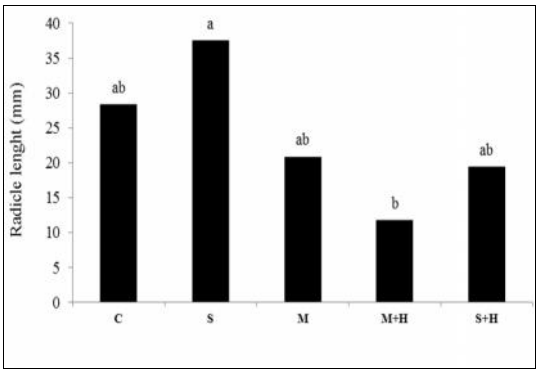

Fig. 7 Effect of different treatments on radicle length of *Kelussia odoratissima* Mozaff.

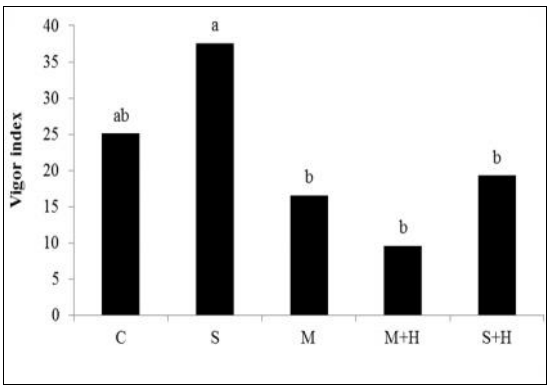

Fig. 8 Effect of different treatments on seedling vigor index of *Kelussia odoratissima* Mozaff.

Under defined conditions it is tested that *P. indica* promotes the germination of orchid seeds and promotes the formation of the protocorm [43].

Also, it seems that *P. indica* culture filtrate is effective in breaking the dormancy, seed germination and enhancement of the seedlings [44]. In *Helianthus annuus* L., *P. indica* culture filtrate was evidenced to influence the seed-oil yield [45]. However, to the knowledge of the author, the effect

of *P. indica* on seed germination of medicinal plant has not been examined before.

Plant-growth-promoting microorganisms, including *P. indica*, produce auxins, which may be active in plants [18,46,47]. Microorganisms also could interfere with the plant auxin synthesis, metabolism, signaling, and transport [16,48] or they affect the plant hormones balance [47].

Although in the past the role of auxin in germination was not necessary, but now by according to the analysis regarding the expression of auxin related genes, its important role has been proven before and after germination. For example, auxin RESPONSE FACTOR 10 during seed germination is prevented by microRNA 60, so that the seed can germinate [49,50]. Interactions with the ABA pathway cause the prevention mechanism [49,50]. It seems that auxin has an important role for the growth of young seedlings [51,52]. The major source of auxin for the seedlings is the stored auxin in the seed cotyledon. The major source of auxin in mature seeds are the amide products [53,54].

Abscisic acid (ABA) and gibberellins are the most fundamental plant hormones for seed germination, which have inhibitory and stimulatory effects on seed germination, respectively. The auxin interactions with gibberellins and ethylene may affect the processes of seed germination and seedling establishment and auxin by itself may not be important for seed germination [55,56].

Seed sensitivity to ABA can increase by alternation of auxin signaling pathway [49,50]. In the presence of ABA, Auxin can effect on seed germination [57]. The molecular mechanism that regulate the interactions and cross-talk between auxin and ABA is not clear yet. By affecting the activity of some enzymes, auxin can also affect seed germination, for example, auxin in germinating pea seeds can regulate the activity of glyoxalase I, resulting in higher rates of cell growth and development [52,58].

Conclusion

According to the results, without moist chilling treatment seed germination in *Kelussia* totally stopped whereas moist chilling caused germination. Application of spore suspension of *P.indica* simultaneously with moist chilling treatment improved different germination traits in *Kelussia* seeds. Although application of spore suspension of *P.indica* significantly increased the final germination percentage, but simultaneous application of gibberellic acid hormone and different treatments of *P.indica* (spore and mycelium) did not have significant effect on the seed germination rate and final germination percentage. For the future experiment application of higher doses of spore and also investigation the effect of *P.indica* on emergence and growth of *Kelussia* seedling in soil condition is recommendable.

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