Romanian Biotechnological Letters *Vol. , No. x,*

ORIGINAL PAPER

Seed germination of some medicinal plant species for conservative purpose

Received for publication, Mat, 23, 2017
Accepted, June, 16, 2018

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Abstract

Due to the overexploitation, many medicinal plants are in decline. Enhancing the seed germination and the vigorous seedlings production is crucial for their cultivation and for ex situ conservation. Seven medicinal plant species were studied concerning seed germination as effect of different treatments (sterilization procedures and stimulation of germination). Seed sterilization with $HgCl_2$ 0.1% for 12 minutes was effective for controlling microbial and fungal contamination in all tested medicinal species. In vitro conditions were efficient for seed germination for 4 species. The stimulation treatments tested (KNO_3 0.1%, NaCl 0.5% and GA_3 0.5%) determined an improvement of seed germination capacity compared with untreated seeds.

Keywords: medicinal plant, germination, stimulation, conservation.

Introduction

The medicinal plant species represent a consistent part of the natural biodiversity. In May 2015, an IUCN report showed that Europe's medicinal plants are in decline due to collection of plants from the wild (for their medicinal value, ornamental and horticultural plant trade) (ALLEN & al. [1]). In our days, ~ 80% of the people use medicinal plant species (crude materials, processed products like pharmaceuticals, herbal remedies, teas, spirits, cosmetics, sweets, dietary supplements, insecticides etc.) for their primary health care (ARCEUSZ & al. [2]).

In România almost 900 plant species represent medicinal flora (MUNTEAN [3]). The research directions in these species were focused on their biology, the content of active ingredients and the improvement of culture technology (MUNTEAN [3]).

Three from the selected species are included in the European Red List of Medicinal Plants like least concern (*Agrimonia eupatoria*, *Cnicus benedictus*, *Geum urbanum*) (ALLAN et al. [1]). The others, *Foeniculum vulgare* and *Hyssopus officinalis* are plants produced at commercial scale. *F. vulgare* is characterised by poor seed germination (TAHAEI et al., [4]).

According to Guidelines on the conservation of medicinal plants [5], the strategies used for the conservation of medicinal plant species applied over the world are represented by *in situ* and *ex situ* strategies. The *ex situ* conservation was classified as *in vivo* (botanical gardens, field cultures, seed banks) and *in vitro* (tissue cultures, artificial seeds, cryopreservation) methods. The plant cells and tissues culture technologies offer a great opportunity to exploit the medicinal plants (SHARMA & al. [6]).

Seed germination and seedling establishment are the initial stages of plant development. These processes are unpredictable over space and time. The germination process is specific for each species, depending on different factors like seed maturity, physical conditions (temperature and humidity), harvesting methods, transport and maintenance. In the case of medicinal plants, there is the possibility that seeds can germinate only in the native environment and fail to germinate under the laboratory conditions (GUPTA [7]). Knowledge of the seeds germination of medicinal plants has proved to be useful in development of appropriate conservation strategies (KANDARI & al. [8]).
Low germination rates (frequently result from fungal infection or mechanical damage) or specific ecological requirements may affect the cultivation of some herbs (VINES [9]). Enhancing the seeds germination and developing of the vigorous seedlings is crucial for an *ex situ* conservation strategy. The first step of *in vitro* cultures establishment is the sterilization - process consisting in different treatments having as effect explants free of contamination; viable decontaminated explants are used further for the initiation of tissues culture. When in case of conventional methods, a low rate or no germination was registered, *in vitro* techniques may enhance the germination process (FAY [10]).
The aim of the present study was to check and improve the seed germination capacity of some medicinal plant for conservative purpose.

Materials and Methods

Mature seeds from different species of medicinal plants belonging to four families (Table 1) were tested concerning the germination capacity.

Table 1. The medicinal plant species tested for seed germination capacity

Plant species	Family	Life cycle	Used for	Seeds aspect
Agrimonia eupatoria L.	Rosaceae	perennial	disorders related to the liver, bile, gastrointestinal, respiratory tract	
Cnicus benedictus L.	Asteraceae	annual	*In vitro* antibacterial activity against *Bacillus subtilis, Brucella species, E. coli, Proteus species, P. aeruginosa, S. aureus, Streptococcus faecalis*, anti-HIV, anticancer agents	
Geum urbanum L.	Asteraceae	perennial	rheumatism, gout, infections, fever	
Coriandrum sativum L.	Apiaceae	annual	cold, seasonal fever, stomach disorders, against worms, rheumatism and pain in the joints	

Foeniculum vulgare Mill.	Apiaceae	perennial	antioxidant, anti-inflammatory, antimicrobial, estrogenic, diuretic, antithrombotic, hepatoprotective, memory enhancing, antimutagenic	
Pimpinella anisum L.	Apiaceae	annual	analgesic, carminative, aromatic, disinfectant, diuretic	
Hyssopus officinalis L.	Lamiaceae	perennial	expectorant, antiseptic	

The seeds of *F. vulgare*, *P. anisum* and *H. officinalis* were bought from market, in 2014 and others (*A. eupatoria, C. benedictus, G. urbanum, C. sativum)* were kindly provided by Nicoleta Constantin in 2012, from Botanical Garden, Bucharest.

All seeds were hand sorted to select undamaged seeds. For germination experiments were used 75 seeds in 3 replications/ each treatment. After treatments, seeds were inoculated in distilled water, on filter paper in Petri dishes in culture chamber at 25°C and a photoperiod of 16/8 and illumination at 1500 lux. Germinated seeds were counted every day. A seed was considered germinated when the tip of the radicle had grown free of the seed coat.

Experiment 1: Seed germination *in vitro* and *in vivo.*

To compare the seed germination capacity in 7 medicinal plant species, *in vitro* and *in vivo* conditions were tested, temperature and illumination regime being the same (25°C and 16/8 hours photoperiod, 1500 lux illumination).

For *in vitro* germination, all seeds were sterilized. Steps of sterilization protocol consisted in washing in running tap water (for 2 - 3 hours), the immersion in ethanol 70º (30 seconds), in the treatment with $HgCl_2$ 0.1%, (for 12 minutes) followed by three washing in sterile distilled water.

For *in vivo* germination, unsterilized seeds were placed on filter paper discs wetted with distilled water in placed in 10cm Petri dishes.

Experiment 2: **Seed germination improvement**.

Among the species analyzed, were chosen two species, one with no germination *in vivo* and low *in vitro* response (*C. sativum*) and one without any positive germination response (*C. benedictus*). For these species, some stimulation treatments were applied to improve the seeds germination. Seeds were maintained in KNO_3 0.1%, NaCl 0.5% and GA_3 0.5% solutions for 3 days at room temperature. The control was represented by seeds cultured on filter paper with distilled water for 3 days without any treatment. No sterilization protocol was used.

After stimulation of germination during 3 days, the seeds (treated and untreated) were inoculated in a substrate consisted in ground and perilte mix in equal parts. Seeds were periodically wetted with tap water without any growth factors or stimulants.

The recorded parameters were the final germination percentage (mean value ± standard error) and mean germination time calculated after formulas:

Final germination percentage = total number of germinated seeds/total number of seeds X 100.

Mean germination time = Σ (nT)/ ΣN (ELLIS & ROBERTS [11]), where n = number of seeds newly germinated at time T at 25°C; T = time (hours, days) from the beginning of the germination test, N = final germination.
The plants obtained from germinated seeds were further used as sources of explants for *in vitro* multiplication.
The photos were taken with a Panasonic DMC-LS80 camera. The results were statistically analyzed using ANOVA from Daniel's XL Toolbox version 6.52 (http://xltoolbox.sourceforge.net). A posthoc test (Bonferonni-Holm) was applied to check the significant differences among means.

Results and discussion

In the present study, aspects concerning seeds germination in seven medicinal plants were evaluated. In the first experiment, seeds were sterilized. It is known that seeds collected from the natural habitats are often contaminated with exogenous and endogenous microbial contaminants (HALLOIN [12]; AHMAD & al. [13]). The seeds sterilization is a prerequisite for successful *in vitro* germination. Agents as ethanol, sodium hypochlorite, calcium hypochlorite, chlorine gas and mercuric chloride have been widely used for surface sterilization of parts of the plant and seed material of various species (TALEI & al. [14]; DAUD & al. [15]; BARAMPURAM & al. [16]).
In our experiment, although $HgCl_2$ is a toxic agent, at the level used (0.1%) and time of exposure (12 minutes) was effective for controlling microbial and fungal contamination in all medicinal plant species tested. Previous works reported that treatment with $HgCl_2$ at 0.1-0.2% for 10-15 minutes was effective for surface sterilization of other like *Aquilaria agallocha* (HE & al. [17]) and *Aquilaria sinensis* (SHU & al. [18]).
Germination percentage recorded *in vivo* and *in vitro* conditions (Table 2) shows that *in vitro* culture conditions had a stimulatory effect in the case of *Agrimonia eupatoria, Geum urbanum, Coriandrum sativum* and *Pimpinella anisum* seeds.

Table 2. Germination percentage of the tested medicinal plant species *in vitro* and *in vivo* conditions

Medicinal plant species	Treatments	Germination percentage	F	P
Agrimonia eupatoria	*in vivo*	0^a		
	in vitro	40.9^b	57.558	3.20549E-12
Geum urbanum	*in vivo*	0^a		
	in vitro	43.47^b	57.558	3.20549E-12
Hyssopus officinalis	*in vivo*	16.66^a		
	in vitro	0^b	14.062	0.000
Coriandrum sativum	*In vivo*	0^a		
	in vitro	13.63^b	11.363	0.000
Foeniculum vulgare	*In vivo*	44^a		
	in vitro	$7,14^b$	28.971	2.77284E-07
Pimpinella anisum	*In vivo*	0^a		0,012254
	in vitro	8.82^b	6,428	
Cnicus benedictus	*In vivo*	0		
	in vitro	0		

Values having the same letter are not significantly different at 0.05 significance level.

The final germination percentage varied between 7.14% for *F. vulgare* and 43.4% for *G. urbanum.* Using Bonferonni-Holm test showed significant differences between sterilized and unsterilized seeds in the case of *Hysopus officinalis, Coriandrum sativum, Foeniculum vulgare* ($p<0.0002$), *Pimpinella anisum* (at $p<0.01$) (Table 2). Seeds of *Cnicus benedictus* did not germinated in the first experiment.

This lower germination percentage could be attributed to factors such as lack of nutrients, seeds being inoculated in distilled water without growth factors or germination enhancing factors.

Seeds inoculated *in vivo,* at the same temperature, humidity, illumination and substrate, but without sterilization, did not germinate in five tested species. Only in the case of *F. vulgare* (44%) and *H. officinalis* (16.66%) the seeds germinated.

Our results concern the efficiency of *in vitro* conditions than *in vivo* are in accordance with studies about *Cassia alata* seed germination (THIRUPATHI & al. [19]). *Asparagus racemosus* (RAGHAV & KASERA [20]) and *Alstroemeria ligtu* hybrid (NASRI & al. [21]) seeds germination was improved by *in vitro* conditions.

In the case of fennel seeds, it was demonstrated that the optimum temperature for germination is around 22ºC with small differences for each variety (THOMAS [22]). It was previously reported that the fennel seeds need some treatments to increase the germination rate (NEACȘU *&* al. [23]). In our experiment, seed germination was low both *in vivo* (44%) and *in vitro* (7.14%) (Table 2). In the case of this species, is possible that sterilization with $HgCl_2$ inhibited the seed germination.

Usually, *Agrimonia eupatoria* seeds germinate in 2-6 weeks at 13°C (RICE [24]). In our experiment, seeds were collected in 2012 and were stored at room temperature for 2 years. No seed germination was observed at 25°C, neither control seeds nor seeds treated with stimulatory treatments. After *in vitro* procedures (sterilization with ethanol and $HgCl_2$ at 25°C) 40.9% of *Agrimonia* seeds germinated. An explanation of *in vitro* seed germination in this species might be that sterilization with ethanol and $HgCl_2$ break the dormancy of zygotic embryos. These results are accordingly with others authors (JEAVONS & JARVIS [25]) which used a treatment with ethanol and mercuric chloride to break the dormancy of intact hazel seeds.

In the second experiment, the effects of stimulatory treatments (KNO_3, NaCl and GA_3) applied *in vivo* were analyzed (Table 3). The stimulation treatments have generally been applied to enhance seed germination (JOSHI & DHAR [26]) and to increase seedling vigor (LEITE & al. [27]).

Table 3. Seeds germination and applied treatments for *C. benedictus* and *C. sativum.*

Medicinal plant species	Treatments	Mean germination
Cnicus benedictus	Control	0 ± 0^{a}
	KNO_3 0.1%	0.671 ± 0.47^{b}
	NaCl 0.5%	0.315 ± 0.46^{c}
	GA_3 0.5%	0.644 ± 0.48^{c}
Coriandrum sativum	Control	0 ± 0^{a}
	KNO_3 0.1%	0.671 ± 0.47^{b}
	NaCl 0.5%	0.63 ± 0.44^{c}
	GA_3 0.5%	0.329 ± 0.47^{c}

Values having the same letter are not significantly different at 0.05 significance level.

In our experiments, the treatments with KNO3, NaCl or GA_3 were effective in improving seed germination. Seeds of *Coriandrum sativum* exposed at KNO_3 0.1% and GA_3 0.5% showed an improved germination capacity (68%) comparing with control. In the case of *Cnicus benedictus* the highest final germination percentage (67%) was recorded for seeds soaked in 0.5% GA_3. A lower germination (33.33%) was recorded in the case of seeds treated with 0.5% NaCl. The results showed that there are significant differences between treated and untreated seeds for both species *C. sativum* and *C. benedictus* (Table 4).

Table 4. Result of posthoc test, showing the significance differences between stimulation treatments (using Daniel's XL Toolbox version 6.52).

		Coriandrum sativum		***Cnicus benedictus***	
		Critical	**P**	**Critical**	**P**
Control	KNO_3 0.1%	0.008	0.000	0.008	0.000
Control	GA3 0.5%	0.01	0.000	0.01	0.000
Control	NaCl 0.5%	0.016	0.000	0.0125	0.000
KNO_3 0.1%	NaCl 0.5%	0.012	0.000	0.016	0.000
NaCl 0.5%	GA3 0.5%	0.05	0.377	0.025	0.000
KNO_3 0.1%	GA3 0.5%	0.025	0.000	0.05	0.734

There are several studies concerning seed germination improved with KNO_3 and NaCl solutions in some medicinal plants (GUPTA [7]; SHIVKUMAR & al. [28]; KAMBIZIA & al. [29]; KULKARNI & al. [30]; FARIMAN & al. [31]).
The shortest mean germination time was registered in the case of *Cnicus benedictus* treated with 0.5% GA_3 (13,5 days). With the same treatment, seeds of *Coriandrum sativum* starts to germinated after 16 days. After treatment with 0.5% NaCl, germination capacity starts after 36 days.
Our results are in accordance to other studies which showed that potassium nitrate treatment increased seeds germination in *Avena fatua* (HILTON [32]), *Hypericum avicullariifolium* (ÇIRAK & al. [33]), chicory (TZORTZAKIS [34]), *Foeniculum vulgare* and *Cuscuta epithymum* (TAVILI & al. [35]).

Conclusion

Our results showed that controlled conditions assured by *in vitro* techniques were efficiently for seed germination for almost species tested. Seeds of *Foeniculum vulgare* may be sensible at $HgCl_2$, *in vitro* germination being inhibited. *Cnicus benedictus* seeds did not germinate *in vivo* and *in vitro* conditions, only stimulatory treatments determined an improvement of the germination capacity. Treatment with KNO_3 positively influenced the germination of *Coriandrum sativum* and *Cnicus benedictus*.

Acknowledgements

The study was funded by project no. RO1567-IBB06/2015 from the Institute of Biology Bucharest of Romanian Academy and is part of the Master degree thesis of Miss Lazăr Mădălina.

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