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Biotechnological approach for *ex situ* conservation of the vulnerable species *Moehringia jankae*

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Abstract

In the endemic and vulnerable taxon Moehringia jankae, a micropropagation protocol for ex situ preservation purpose was established. In vitro cultures were started from aseptic germinated seeds. Single or combinated treatments were used, M. jankae seeds being extremelly contaminated. The most effective sterilization agent was 2.7% sodium dichloroisocyanurate with a germination rate of 22%. Different combinations of plant growth regulators (PGRs) were tested to establish the optimum micropropagation protocol using as inocula double node stem cuttings detached from the sterile seedlings. Regeneration through axillary shooting was assessed after one and two months of culture, by evaluating the number of axillary shoots/explant, the number of nodes of the best developed shoot/explant, the maximum length of the best developed shoot/explant. Regeneration occurred on all media tested, but the best response was recorded on MS medium added with 4.4 µM BAP + 0.49 µM IBA with an average of 14 shoots/ explant, registered after two months. In the same interval, rooting occured on all regeneration media, without any special treatment. The regenerated shoots were grown on large Phytatray vessels with MS-PGR free medium.

Key words: aseptic germination of seeds, axillary shoots, regeneration.

1. Introduction

Moehringia jankae Griseb. *Ex* Janka [syn. *Arenaria jankae* (Griseb. ex Janka) Fernald] is an European endemite from the Western area of the Black Sea Region, an element from Dobroudja which can be found both in Romania and Bulgaria. This taxon is considered vulnerable in the Red Book of Vascular Plants from Romania (DIHORU & NEGREAN [1]) and its habitat is protected by DIRECTIVE 92/43/EEC [2].

Moehringia jankae Griseb. ex Janka has 5-11 cm high, with secondary branches, with small, white, delicate flowers. Seeds are very small, black, tuberculate (HALLIDAY [3]). This taxon grows on the craks of rocks with different origin.

In addition to protected area strategy, complementary *ex situ* conservation measures are useful for vulnerable taxa with reduced populations, with low variability, with limited or threatened habitats or with reproduction difficulties. Plant biotechnologies can improve *ex situ* classical strategies providing new options for collection, multiplication and plant biodiversity conservation (CRUZ & al. [4]). *In vitro* plant collection establishment requires the development of short-, medium- and long-term culture protocols (BENSON [5]; CRUZ & al.[4]; PATHAK & ABIDO [6]).

Only few species from *Moehringia* or *Arenaria* genus have been studied mainly in relation to seed germination and/or to *in vitro* propagation (BASKIN and BASKIN [7,8]; KLAVINA & al. [9], PENCE & al. [10]; SORIANO & al. [11]).

Our aim was to elaborate an efficient micropropagation method for the vulnerable taxon *M. jankae*. This *in vitro* protocol complements the traditional conservation measures and can provide plant material for repopulating natural habitats, for cultivation in Botanical Gardens, for basic studies without affecting *in situ* plants.

2. Materials and methods

M. jankae has fragile shoots which rapidly dehydrate when are detached, being difficult to use them as explants. For this reason, seeds are a reliable source for tissue culture initiation.

The seeds were collected in 2014 from a „Natura 2000" site (Cheia Constanţa County) and were vernalized at 4°C for 60 days.

M. jankae seed morphology was analyzed at scanning electron microscope JEOL SEM 6610. Seeds were air dryed, placed on aluminium stubs and coated with gold layer. Non-germinated and germinated seeds were also analysed at stereomicroscope Zeiss Stemi 2000-C.

Seeds sterilization: several procedures were used after two hours of tap water washing and 30 seconds of seeds immersion in 70º alcohol. There were tested: **(I)** one step treatments by seeds exposure to a single sterilization solution and **(II)** two steps treatments by seeds exposure to two subsequent sterilization solutions (Table 1). Each sterilization solution was added with 2-3 drops of Tween 20. All these procedures were followed by three washes with sterile distiled water.

Twenty sterilized seeds were inoculated in a 10 cm diameter Petri dish and for every sterilization procedure three repetitions (3 Petri dishes × 20 seeds) were made.

Seeds were cultured on MS medium (MURASHIGE & SKOOG [12]) supplemented with 0.5 g l^{-1} active charcoal, 20 g l^{-1} sucrose, 5 mg l^{-1}Gibberelic acid, and 8 g l^{-1}Duchefa agar (w/v). The pH of the medium was adjusted at 5.8 before autoclaving at 121ºC for 15 min. The cultures were maintained at 25 ± 1ºC and 60 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by white fluorescent lamps, with 8/16 hrs photoperiod.

The contamination rate was expressed as the number of contaminated seeds/inoculated seeds× 100 and the germination rate as the number of total germinated seeds/number of sterile seeds× 100 for each sterilization variant. The rate of contamination was scored two weeks after inoculation and the germination rate was recorded three weeks after inoculation.

The data results of seed contamination and germination were subjected to arcsine transformation to ensure the homogeneity of variance before to statisic analysis (SNEDECOR and COCHRAN [13]).

Induction of axillary shoots: double node explants (1 cm) were excised from one-month old *in vitro* seedlings and were cultured on nine variants of solid MS basal medium supplemented with B5 Gamborg vitamins (GAMBORG [14]) and 8 g l^{-1}(w/v) agar (Duchefa Biochimie B.V). The eight PGRs treatments were: 4.4 µM 6-benzylaminopurine (BAP)+0.54 µM α-naphthaleneacetic acid (NAA)(MS1), 4.5 µM 6-[4-Hydroxy-3-methil-but-2-enylamino]purine (ZEA) + 0.49 µM indole-3-butyric acid (IBA) (MS2), 4.4 µM BAP + 0.49 µM IBA (MS3), 4.6 µM 6-Furfurilaminopurine or kinetin (KIN) + 0.49 µM IBA (MS4), 271.5 µM Adenine sulphate (Ads) + 2.46 µM IBA (MS5), 0.22 µM (1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea or thidiazuron (TDZ) + 0.049 µM IBA (MS6), 0.045 µM TDZ + 0.049 µM IBA (MS7) and 4.9 µM N6-(2-Isopentenyladenine (2iP) + 0.49 µM IBA (MS8). PGRs-free MS basal medium was used as control (MS0).

The pH of all media was adjusted to 5.8 before autoclaving under 118 kPa and 120°C for 20 min. The explants were cultured in glass jars of 55 mm diameter and 85 mm high, containing 30 ml medium. The cultures were grown in a cultivation chamber at 25 ±1°C with 16 hours of cool and white fluorescent light at 60 μM m^{-2} s^{-1} per day. Four explants were used per treatment and the experiment was repeated three times.

The *in vitro* response of *M. jankae* explants was evaluated after 1 and 2 months assessing: (i) the number of axillary shoots/explant, (ii) the number of nodes of the best developed (tallest) axillary shoot/explant, (iii) the lenght (cm) of the best developed axillary shoot/explant. The results from axillary shooting evaluation were expressed as the mean ± standard deviation (SD).

All data were analyzed using One-Way Anova test (at $p \leq 0.05$). The significance of differences between experimental variants was assessed using a post-hoc test Bonferroni-Holmes at 95% confidence interval (Daniel's XL Toolbox version 6.60 [15]).

Ex vitro acclimatization: well developed regenerated plants (10 cm high) were *ex vitro* transferred for acclimatization in perlite or in a mix of 1:1 parts of perlite and half-strengt MS liquid medium (method adapted after CLAPA & al.[16]) using 20 plants/ substrate variant. The experiment was repeated twice.

During the first two weeks after the transfer, the plants were covered with plastic caps to maintain a high humidity and they were 10 minutes ventilated once/day. The perlite substrate was wetted once per day with half-strength MS liquid medium. The *ex vitro* plants were grown at 25ºC and 60 μmol m^{-2} s^{-1} photosynthetic photon flux density provided by white fluorescent lamps, with 8/16 hrs photoperiod. Subsequently, the humidity was gradually decreased by prolonging the removal of the vessel caps (30 min,1 h, 2 hrs, 4 hrs, 8 hrs/day).

The survival rate of the acclimatized plants was recorded after three weeks as the number of plants resisting without protection against water loss.

3. Results and discussion

In vitro seed sterilization and germination.

M. jankae shoots are sensitive to desiccation, so only seeds are appropriate to harvest and to use for *in vitro* culture establishment and multiplication. For this taxon, seeds sterilization represents a challenge because they having a high level of contamination.

Seed germination represents an important developmental step for *in situ* species survival and *ex situ* preservation approaches. Seeds dormancy is a physiological mechanism to overcome the unfavourable conditions during the winter, its breakdown and germination requirements are peculiar for every taxon, depending on species phylogeny, distribution, habitat and life cycle (VANDERLOCK & al. [17]). For *in vitro* culture, treatments made to ensure seeds sterilization may interfere or even inhibit germination, for this reason being necesary to find the most suitable procedure.

Moehringia jankae seed morphology corresponds to the Eastern-Balkan *Moehringia* group, having lateral faces convex, reniform, the coat has round cells, with 3-4 teeth on each side with irregular contour, the outer surface being formed by mamillate epidermal periclinal walls (MINUTO & al. [18]). *M. jankae* seeds observation at the stereomicroscope (Fig. 1A) and scanning electron microscope (Fig. 1B) beside coat typical irregular shaped cells, also revealed a complex structure called elaiosome.

The elaiosome (strophiole) is originated from the proliferation of white cells of the funiculus corresponding to papillate type (MINUTO & al. [18]) playing a significant role in myrmecochory, the reproductive strategy of mutualism between plant and ants, which is characteristic for taxa which grow in rock cracks or soil over the rocks (SORIANO & al. [11]). It represents a nutritional source for the ants owing to lipids, proteins and sugars content, which contributes to seeds spreading and germination on a suitable substrate (GILADI [19]; CLARK and KING [20]).

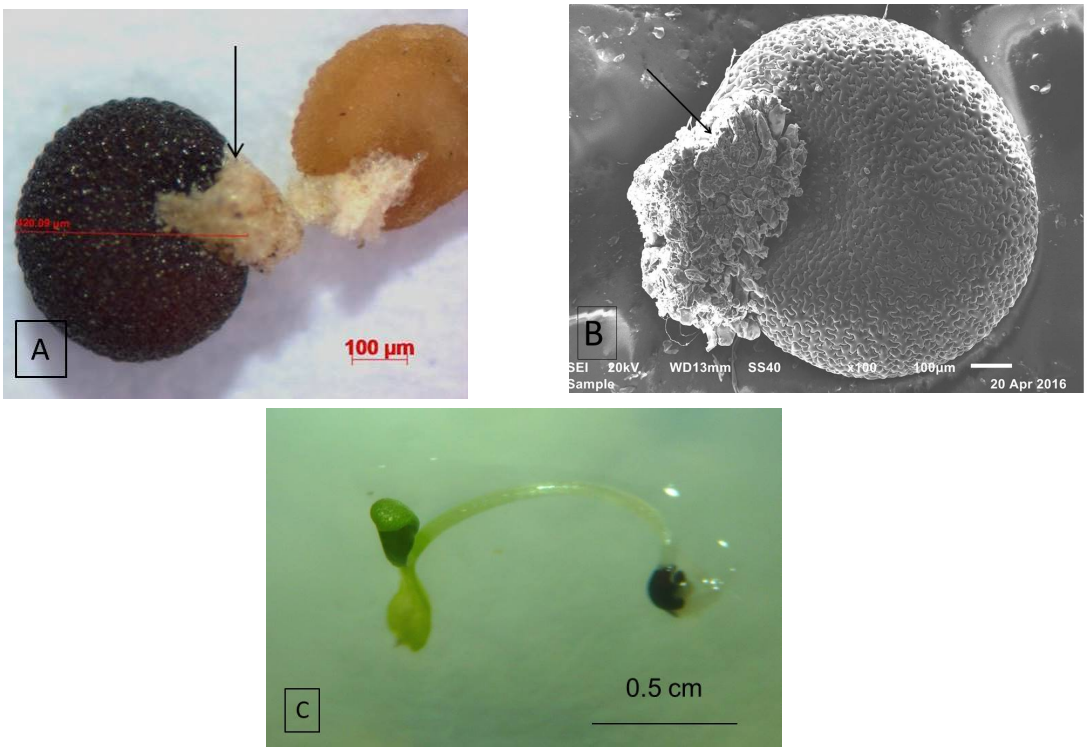

Figure 1 A. Mature (black) and immature (light-brown) seeds of *M. jankae* with elaiosome (black arrow); **B.** *M. jankae* seed morphology with elaiosome and coat features revealed by scanning electron microscopy; **C.** Aseptic germinated seed after 2 weeks from inoculation.

In fact, *M. jankae* seed structure, elaiosome composition and the reproductive strategy connected with ants also favour high seed infection with bacteria and fungi.

Concerning germination, preliminary studies (data not published) showed that the temperature variations (10-20°C) and the illumination regime (dark or light) did not influence the germination rate of the 60 days-vernalized *M. jankae* seeds at 4°C.

Using different treatments, seed contamination rate varied depending on the active compounds used, between 0% (in the case of mercuric chloride) and 75% (in the case of sodium hypochorite and hydrogen peroxide alone) (Table 1). The seeds started to germinate after 7-10 days (Fig. 1C) and continued during two weeks.

In *M. jankae*, mercuric chloride inhibited seeds germination, while hydrogen peroxide did not efficiently disinfect the seeds even after two treatments. Generally, the germination rate was quite reduced. Despite increased seed contamination, the maximum germination rate recorded in our testing was 22% in the case of Pursept use (2.7% dichloro isocyanurate) as disinfection agent. In fact, sterilizing agents decrease or inhibit seeds germination.

In comparison, for some related threatened *Dianthus* species, seed sterilization were more effective as in *M. jankae*: 42% in *D. giganteus* subsp. *croaticus*, 70 % in *D. ciliatus* subsp. *dalmaticus* (RADOJEVIĆ & al. [21]), 97% in *D. serotinus* (MARKOVIĆ & al. [22]), 92% in *D. pinifolius* (MARKOVIĆ & al. [23]), 75% in *D. henteri* and *D. nardiformis* (CRISTEA & al. [24]; HOLOBIUC & al. [25]). In contrast, only few studies on *Moehringia* or *Arenaria in vitro* germination were carried out, germination rate being generally low.

In *Arenaria fontinalis,* an endemic close-related taxon from USA (BASKIN and BASKIN [8]), the non-sterile seeds were postmaturated in the greenhouse using different temperature treatments and stratification and germinated after a long period of time in soil, just the following autumn.

In *M. trinervia,* a low germination rate (7-8%) induced in non-sterile conditions by temperature variation and nitrates addition was also reported (VANDERLOCK & al. [26]).

In the chasmophytic mediteranean species *M. papulosa,* for seeds collected from several natural populations, the germination average were 20%, 15%, 10%, just in case of devoid-elaiosome seeds (SORIANO & al. [11]). In this study, treatment made in non-sterile conditions with gibberellin (0.722 mM) and scarification improved germination at 60%.

In the relative *Minuartia cumberlandensis (*PENCE & al. [10]), for the seeds collected from two different populations, after sterilization using commercial bleach diluted 1:20 for 5 minutes was obtained an average of seed viability varying between 12% and 50%.

The viability and seed germination depend on the site and its climatic and ecological features, a high correlation between seed germination requirements and habitat type connected to the amount of rain was reported (BASKIN and BASKIN [7]; VANDERLOCK & al. [26]; SORIANO & al. [11]).

The use of appropriate seeds sources and the possibility to improve seeds germination response is important for conservation programs of plants growing in specific habitats (PRADHAM and BADOLA [27]). For *in vitro* culture establishment, this aspect had to be combined with an appropriate, non-inhibitory sterilization method able to ensure a satisfactory number of sterile and viable seeds.

In *M. jankae*, the seeds were originated from the population Cheia (Constanța County) and their contamination and germination depends on the peculiarities of the site (rock cracks in the Jurassic recife structures) and on the annual climate characteristic when seeds were collected.

Table 1. The treatments used to sterilize *M. jankae* seeds, the contamination and total germination rate.

Treatments used			**Contamination (%) ± SD**	**Germination (%)± SD**
Var.	**I**	**II**		
1.1	Mercuric chloride 0.1% 10 mins	-	**0** ^{a}	**0**^{a}
1.2	Thimerosal (sodium ethylmercurithiosalicylate) 0.1% 10 mins	-	**10±1.51**^{b}	**14.2±2.69**^{b}
2.1	Hydrogen peroxide 2.5 % 15 hrs	Hydrogen peroxide 10% 10 mins	**75±2.44**^{cd}	**0**^{a}
2.2	Hydrogen peroxide 4 % 12 hrs	Hydrogen peroxide 10% 20 mins	**25±12.24**^{bd}	**5.5±0.40**^{cd}
3.1	Sodium dichloroisocyanurate 0.5 % 10 mins	-	**25±4.08**^{bc}	**7.7±1.83**^{bd}
3.2	Pursept (Sodium dichloroisocyanurate 2.7 %) 10 mins		**10±1.63**^{b}	**22±1.63**^{b}
4.1	Domestos ½ (Sodium hypochlorite 2,5%) 15 mins	-	**10±0.00**^{bd}	**10±4.08**^{bd}
4.2	Ace 15% (< 5% Sodium hypochlorite) 20 mins	-	**75±4.08**^{cd}	**0**^{a}
5	Domestos ½ (Sodium hypochlorite 2,5%) 5 mins	Sodium dichloroisocyanurate 0.5 % 10 mins	**25±4.08**^{b}	**7.8±4.08**^{bd}

Values marked with the same letter are not significantly different (mean values ± SD at p≤0.05).

Induction of axillary shoots.

Generally, cytokinins determine the differentiation process, auxins promote dedifferentiation and rooting, while combining the two induces multiple axillary shooting at the level of lateral meristems. This ensures the clonality of plant material without genetic variations (KARP [28]).

M. jankae showed a positive *in vitro* response after one month of culture. Axillary shoots formation was induced on all media variants tested, small significant differences among experimental variants concerning regeneration and growth was recorded (Table 2).

Table 2. Evaluation of regeneration of axillary shoots and growth after one month.

Medium variant	Number of axillary shoots/explant	Number of nodes of the best developed shoot/explant
MS0	3.33 ±1.55 ^{bc}	3.5±0.67^{ba}
MS1	3.66 ±2.80^{bc}	3.08±2.91^{ba}
MS 2	3.00 ±1.47^{b}	2.41±2.45^{a}
MS 3	4.16 ±1.52^{c}	3.25±3.27^{ba}
MS 4	3 ±1.75^{b}	3.83±3.73^{ba}
MS 5	2.25 ±0.96^{a}	3.16±3.09^{ba}
MS 6	4.58 ±2.02^{c}	2.5±2.45^{a}
MS 7	3.00 ±1.70^{b}	3.5±0.64^{bc}
MS 8	4.00 ±2.33^{c}	3.08±2.91^{bc}

Values marked with the same letter are not significantly different (mean values ± SD at $p \leq 0.05$).

The tissue cultures viability was good in all tested variants (90-100%) and axillary shoots were formed on all cultured explants (100% regeneration rate).

The best regenerative responses were registered in the presence of 0.22 µM TDZ + 0.049 µM IBA (MS6), 4.4 µM BAP + 0.49 µM IBA (MS3) and 4.9 µM 2iP + 0.49 µM IBA (MS8).

Zeatin at 4.5µM and thidiazuron used at 0.22 µM in combination with IBA at 0.049 µM promoted more compact shoots development.

The number of nodes of the best developed axillary shoot/explant recorded after one month was similar on almost all tested media (Table 2), just in case of KIN use (MS4), the shoots grown more. In the first month, rooting was not observed on variants with BAP and NAA, KIN and NAA, or TDZ and IBA, while in the presence of BAP and IBA or 2iP and IBA, some spontaneus roots were developed. Zeatin combined with IBA also determined a reduced roots development.

Although NAA and IBA alone are known to promote elongation and rhizogenesis (DE KLERK [29]), in the tested variants (contaning dominat cytokinins and less auxins), rooting of the axillary shoots in the first month of culture was favored just by certain combination of cytokinins/auxins.

After two months of culture, signifficant differences on the *in vitro* response were recorded (Table 3), this time-interval being suitable to evaluate the best PGRs treatment for micro-propagation improvement.

Shoots regeneration was increased on almost all media tested (Fig. 2), producing over 30 axillary shoots/explant in presence of BAP and IBA (MS3), zeatin induced also a good regenerative response with compact shoots and floral buds (Fig. 2A), but the shoots growth was limited. The lowest response was recorded in the presence of 271.5 µM Ads and 2.46 µM IBA (MS5). Adenine sulphate did not improve the regeneration, only few long shoots were developed (Fig. 2F). Combination of 4.4 µM BAP and 0.49 µM IBA (MS3), 4.5 µM ZEA and 0.49 µM IBA (MS2) and 0.22µM TDZ and 0.049 µM IBA (MS6) determined an increased number of axillary shoots after two months, while in presence of KIN 4.6 µM/ IBA 0.49 µM

(MS4), TDZ 0.045 µM /IBA 0.049 µM (MS7) and 4.9 µM 2iP /0.49 µM IBA (MS8) the number of axillary shoots was more reduced. Thidiazuron used at 0.045 µM determined the etiolation of the shoots in the second months of culture.

Table 3. Evaluation of regeneration of axillary shoots and growth after two months.

Medium variant	Number of axillary shoots/explant	Number of nodes of the best developed shoot/explant	Length of the best developed shoot/explant
MS0	4.08±1.16^{a}	6.83±1.11^{c}	12.94±1.29^{c}
MS1	4.16±2.88^{a}	3.66±2.01^{a}	3.94±1.10^{a}
MS 2	13.58±9.82^{d}	3.75±0.62^{a}	3.93±0.84^{a}
MS 3	14.16±11.39^{d}	4.00±1.046^{a}	4.95±1.47^{a}
MS 4	5.66±2.60^{c}	6.16±1.40^{c}	7.7±2.25^{b}
MS 5	1.91±1.24^{b}	4.83±1.02^{b}	7.92±3.07^{b}
MS 6	13.41±15.15^{d}	3.33±1.82^{a}	4.65±3.78^{a}
MS 7	5.91±4.01^{c}	4.83±1.19^{b}	6.87±1.98^{b}
MS 8	6.00±3.35^{c}	5.91±0.99^{c}	7.64±1.97^{b}

Values marked with the same letter are not significantly different (mean values ± SD at p≤0.05).

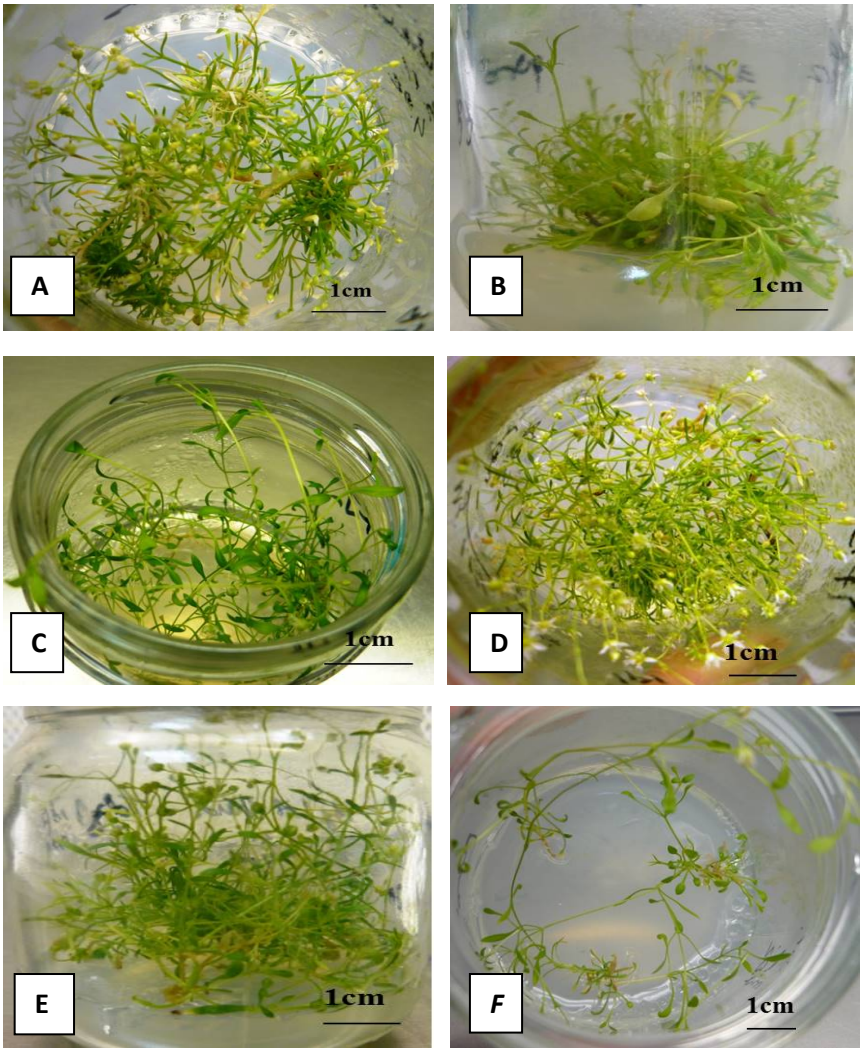

Figure 2. *In vitro* regenerative response induced after two months of culture on medium supplemented with combination of: **A.** ZEA and IBA (MS2); **B.** BAP and IBA (MS3); **C.** KIN and IBA (MS4); **D.** TDZ and IBA (MS6); **E.** 2iP and IBA (MS8); **F.** Ads and IBA (MS5).

After two months of culture, KIN, Ads, and 2iP combined with IBA, determined differences in shoots length in the case of MS4, MS5, MS7 and MS8 media.

Regenerated axillary shoots developed more concerning the number of the nodes in the case of MS4 and MS8 variants. In comparison with control (MS0), the growth of the shoots was lower on all regeneration media. After eight weeks, the axillary shoots rooted on all regeneration media or on MS-PGR-free medium, no specific treatments being necesary and no relevant differences among rooting respose being recorded (100% rooting).

Also an intense *in vitro* flowering process occurred using zeatin or thidiazuron (MS2 and MS6) (Fig. 2A, D) and a less intense in the presence of Ads (Fig. 2F). *In vitro* flowering is not a common phenomenon (TAYLOR & al. [30], ZIV and NAOR [31]), that can occur spontaneously or may be induced by zeatin, 2iP, and TDZ as we observed in *M. jankae*.

Concerning cytokinins/auxins ratio, good regeneration was reported in related *Dianthus* threatened taxa when the ratio was 10:1 (CRISTEA & al. [32, 24, 33]; JARDA & al. [34]; HOLOBIUC & al. [35, 25, 36]), 5:1 and 2:1 (MARCOVIĆ & al. [37, 23]), 2:1 (MARCU & al. [38]).

As an exception, in the case of *Dianthus mainensis* (ERST & al. [39]), just 3-5 µM BAP alone promoted regeneration at the best rate.

In the close relative, *Arenaria procera*, the proliferation rate was positive, but lower (1.3) in the presence of BAP and rooting occured easily (KLAVINA & al. [9]).

In *Minuartia valentina*, young rametes were used as explant source and cultured on different media with three cytokinins (BAP, KIN, 2iP) at four concentrations (0.2, 0.5, 1, 1.5 mg l^{-1}) (IBANEZ and AMO-MARCO [40]), despite good regenerants survival and viability, the regeneration (mean number of shoots taller than 10 mm/explant) was quite low (1.4 /explant), induced in the presence of KIN and phloroglucinol (at 80 mg l^{-1}). Elongation was also reduced, occuring in the presence of phloroglucinol and rooting was induced just in the presence of different auxins (IBA, NAA, IAA).

For *Minuartia cumberlandensis* micropropagation, the ratio of PGRs used (BAP and NAA) was 10: 1, aseptic seedling shoots being used as explants. BAP at 1 or 0.5 mg l^{-1} and NAA at 0.01 or 0.05 mg l^{-1} were added to induce axillary shooting with a lower rate of 2-5 shoots (PENCE & al. 2011). *M. jankae in vitro* behavior was similar with other related species from *Dianthus* genus previously tested by us (HOLOBIUC & al. [35, 25, 36]) with some peculiarities about *in vitro* flowering and rooting. Compared to the regeneration reported in the close-related taxa, *M. jankae* regeneration was more efficiently and rooting occurred easily (Fig.3A).

Regenerants acclimatization was achieved on a semisolid mixture of 1:1 parts of perlite and half-strength MS liquid medium (method adapted after CLAPA & al. [16]), acclimated plants were transferred into pots (Fig.3B). 50% survival rate was recorded, this taxon being extremely sensitive to *ex vitro* adaptation. On perlite variant the plant survival was reduced at 30%. For this reason, further studies on acclimatization improvement are needed.

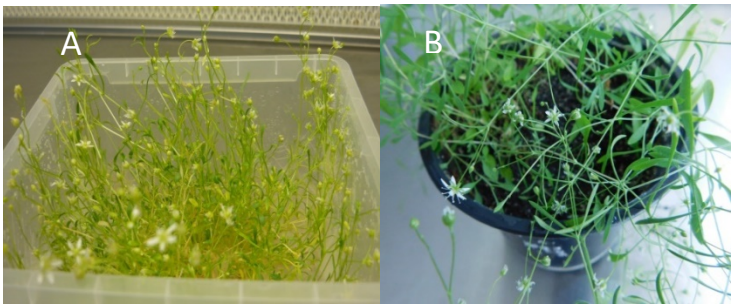

Figure 3 A. Regenerated plants of *M. jankae* on MS-PGRs free medium,
B. *M. jankae* plant transferred on perlite- soil mixture after acclimatization.

4. Conclusions

In *M. jankae*, an optimal micropropagation was obtained starting from aseptic seedlings. Owing to seed morphology and plant myrmecochory, seeds were highly contaminated and germination rate was low. Just sodium dichloroisocyanurate at 2.7% level was more efficient for seeds sterilization. Micropropagation can be optimally made through multiple axillary shooting using 4.4 µMBAP /0.49 µM IBA, 4.5 µMZea /0.49 µM IBA or 0.22 µM TDZ /0.49 µM IBA as PGRs. For a cost-effective micropropagation protocol of *M. jankae*, BAP combined with IBA is recommended to be used.

After two months of continuous culture on the same regeneration media (MS0-MS8 variants), roots were developed on all experimental variants (100% rooting rate) without any special stimulating treatment. Acclimatization was made on semisolid mixture of 1:1 parts of perlite and half-strength MS liquid medium with 50% survival rate.

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