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Influence of seed provenance on the propagation of *Picea abies* (L.) Karst

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Abstract: *Picea abies* (L.) Karst. is the most common species cultivated in Italy for sale as Christmas trees. The aim of this study was to identify the best seed provenance to improve plant production. Three Italian provenances - Gran Bosco di Salbertrand, Pezzel e Fochino, and Val di Fiemme - were examined. A part of seeds were sown on benches in a greenhouse after cold stratification, and another part was used for induction of somatic embryogenesis. In the first experiment, two different amounts of organic matter (manure), as components of growing media, were evaluated, while in the second one different levels and combinations of growth regulators in the medium were tested. The seed provenance and composition of the growing medium influenced seed germination and seedling growth. The best performance on both growing media was achieved by the Val di Fiemme provenance, and in general seedling emergence and growth were the most favourable in the growing medium with a lower organic matter amount. Also, the *in vitro* cultures evidenced differences in the efficiency of somatic embryogenesis among the provenances.

1. Introduction

Norway spruce [*Picea abies* (L.) Karst.] is an economically important tree species that plays a key role in natural ecosystems in the boreal zone from Norway to Siberia, and is one of the most valuable European forest tree species. The natural distribution of *P. abies* in Italy spans the entire Alpine range, where it ascends to an altitude of 2,000 m, and it is also found in part of the northern Apennines. A large portion of the spruce forests are used commercially and managed with varying levels of intensity. In Italy, this species is also the most cultivated for the production of Christmas trees for its conical shape, compact, intense green foliage, and rapid growth. The Christmas trees present on the Italian market are certified because they derived from 90% cultivations in forest nurseries and the trees are usually obtained by seed. Seeds are collected from selected stands, and the obtained seedlings are cultivated in a nursery for almost four years: two years as "seedlings" in seedbeds, and two to three years as "transplantations" in fields depending on the desired size. Christmas tree cultivation is concentrated mainly in Veneto and Tuscany (Arezzo and Pistoia districts) where about 800 hectares are devoted to this crop. Nevertheless, a considerable amount of the seedlings are imported from Belgium, Denmark, Holland, and Germany for the establishment of Christmas tree plantations. Recently, increased importation from Hungary, Romania and Moldavia has evidenced problems related to the genetic control of propagation material. Thus, to avoid genetic mixing between autochthonous species and those coming from abroad, the imported seedlings carry a special label which certifies their origin from specialized cultivations, nationality, and indication as not intended for reforestation.

A sustainable Christmas tree market should aim to increase the production of plants from autochthonous species characterized by high levels of germinability and growth, and to produce seedlings of adequate size and quality in a short time for transplantation.

The commercial importance of genetic characteristics and origin of the propagation material for plantation quality are well documented. In particular, rates of early growth and morphological and physiological characteristics are among the features that differ between populations. Consequently, the provenance of seeds in relation to the choice of planting site is extremely important. Furthermore, identification of both the best seed provenance and environmental conditions for germination and growth of seedlings could be the first objective to improve nursery production. The influence of provenance has not been widely reported on seed germination of *Picea*, while it is well documented for seedling growth. At provenance level, strong relationships are gener-

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ally observed between traits that characterise the duration of the growth period and the degree of lignification. Variation in bud flushing and initiation of shoot growth among plants derived from different Norway spruce seed provenances is assumed to be regulated both by differential responses to the accumulated temperature sum in spring and by conditions during acclimation in the preceding year (Schmidt-Vogt, 1976; Dormling, 1982). Accordingly, trees from with northern latitude or high altitude provenances, which are adapted to a short growing season, have inferior growth potential compared with those adapted to a longer growing season. Variation between plants from different provenances has been demonstrated for a number of other traits, such as nutrient demands, respiratory activity, shade tolerance, and differences in crown shape, and resistance to snow and ice damage (Schmidt-Vogt, 1976).

On the other hand, seedling morphological characteristics before planting were found to highly affect seedling growth during the first years after transplantation (Tsakaldimi, 2006). Furthermore, fertilizer application during two years improved survival and increased annual height of *Betula pubescens* and *Larix sibirica* (O´Skarsson *et al*., 2006). Many studies have reported the effect of manure on increase diameter and height growth of some species of *Pinus* (Nourshad and Ghorani, 1990), and biomass (root and shoot dry weight) of potted and bare rooted *P. taeda* seedlings (Kiani *et al*., 2005). Moreover, combinations of types of soil in different ratios of nutrients have also influenced seed germination of important forest species (Selivanovskaya and Latypova, 2006). Germination, survival, growth, and biomass of *Cupressus arizonica* and *Cupressus sempervirens* are enhanced using organic matter and in particular, manure has increased the maximum quality index (Dickson *et al*., 1960; Ahmadloo *et al*., 2012).

Therefore, the aim of this study was to assess the influence of seed provenance on emergence and seedling growth of *Picea* and, as an ancillary purpose, to evaluate if an increase of the amount of organic matter (dust manure) in the growing media compared to that commonly used can improve or affect the emergence and seedling growth of *Picea* seeds of different provenance. Further, considering that somatic embryogenesis is potentially the most promising method for vegetative propagation of coniferous genera (Dunstan *et al*., 1995), the influence of seed provenance has also been evaluated on somatic embryo germination starting from embryogenic tissues.

2. Materials and Methods

Seed lots

The seeds were provided by the National Centre for the Study and Maintenance of Forest Biodiversity (CNBF) of Peri (Verona, Italy). CNBF is primarily involved in the production, selection, and conservation of forest seeds (of trees and shrubs). Specifically, the forest seeds produced by the Centre come from forests enrolled in the "Libro Nazionale Boschi da Seme" (L.N.B.S.), and traditional and "special" harvesting methods are used in order to preserve the genetic diversity of the species. All the material is certified and traceable through Global Positioning System (GPS) technology (http://www3.corpoforestale.it). In this study, mature seeds of *Picea abies* (L.) Karst. harvested in October 2011 from selected populations of the following three Italian provenances were evaluated: Gran Bosco di Salbertrand - GS (L.N.B.S n. 088, CSR n. 034-Pie; 45°N; altitude 1030-1900 m); Pezzel e Fochino - PF (L.N.B.S n. 137 SO 037; 46°N; 1350-1540 m); and Val di Fiemme - VF (L.N.B.S n. 023 Val di Fiemme TN; 46°N; 1600-2000 m).

Certification of seed quality (purity, weight of 1000 seeds, germinability, cut test, viability, value crop) in accordance with the ISTA (International Seed Testing Association) was carried out by CNBF and is reported in Table 1.

In vivo experiment

The seeds from each provenance were stored separately in plastic bags at 5°C. Later, two sowing beds (each 1.60 m × 5.0 m) were prepared in a greenhouse. A common nursery soil, composed of a mixture of 75% sand, 10% perlite, and 15% organic material (mixture of peat, manure) for a total organic C content of 6.26%, was utilized for the first bed (Substrate 1). The same substrate with a rate higher than one-and-a-half of organic material (total organic C 9.82%) was used for the second bed (Substrate 2) (Table 2). Chemical parameters of growing media were determined according to the SISS methods (SISS, 1985), whereas the ones of organic carbon (OC), nitrogen (N), and inorganic carbon (IC) by using NA 1500 CHNS Analyzer, Carlo Erba (Milan, Italy) coupled with the procedure reported by Santi *et al*. (2006). In March 2012, an average of 20 and 15 g seeds, respectively, for GS, PF, and VF provenances were sown on each sowing bed. Before the trials, the seeds were soaked in water for 12 h. The seeds were sown in seven rows, and a thin layer

Table 1 - Certification of seed quality

Seed provenance	Purity (%)	Weight 1000 seeds	Germinability (%)	Cut Test (%)	No. viable seeds/g	Value crop (%)
Gran Bosco Salbertrand (LNBS n.088 (SR n.054- Pie.)	94.5	7	71	91	96	67.1
Pezzel e Fochino (LNBS n.137 SO037)	92	6.4	74	88	113	68.1
Val di Fiemme (LNBS n.023 Val di Fiemme)	97.2	8.8	92	97	119	89.4

Table 2 - Chemical characteristics of growing media

Growing media	Substrate 1	Substrate 2
C [(z)] (%)	6.26	9.82
Organic matter %)	10.91	17.25
N [(y)] (%)	0.2	0.39
C/N	31.3	25.17
IC [(x)] (%)	2.83	1.47
CEC [(w)] ($mol_c kg^{-1}$)	14.4±2.7	32.0±3.9
pH	7.3±0.2	7.1±0.2

[(z)] C= Organic carbon;
[(y)] N= Nitrogen;
[(x)] IC= Inorganic carbon;
[(w)] CEC= Cationic exchange capacity.

of sand was applied to cover the seeds. The sowing beds were watered as necessary. After emergence, the seedlings were fertilised twice at intervals of 15 days with 50 ppm Flory 4 (Agrochimica). Emergence rates were recorded in March, April, and May. The final stem height (cm) was determined for all seedlings at 90 days, the time of their transfer to pots. In June 2012 the seedlings were transferred to pots (volume 80 ml) filled with the same growing media. Two hundred seedlings per provenance were distributed in five multipot PVC pots of 40 places each. The seedlings were transferred to a shaded (50% light reduction) location outdoors and cultivated until the end of July 2013. Growth was evaluated after 4, 12 and 52 weeks. On the final date, 15 seedlings per provenance were randomly selected for root and shoot biomass measurements. The fresh weight (FW) and dry weight (DW), obtained by drying in an oven at 70°C for 48 h, were determined. The root-to-shoot DW ratio was also calculated for each seedling.

In vitro experiment

For induction of embryogenic tissues, the seeds were sterilized in 2% (v/v) sodium hypochlorite solution for 20 min and rinsed three times with sterile distilled water. The zygotic embryo was dissected from each seed and placed on solid BM1 medium (Gupta *et al*., 1987) supplemented with 1% sucrose, hydrolysed casein (500 mg/l), L-glutamine (450 mg/l), and myo-inositol (1g/l), and solidified with 0.3% Phytogel. Five or 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 2.5 or 5 μM 6-benzyladenine (BA) were included in the medium. The trial was carried out in Petri dishes (100 mm × 15 mm) with 40 embryos per provenance, divided into five replicates of eight embryos each. The embryos were incubated in the dark at 20°C. The cultures were assessed after incubation for four weeks.

For culture proliferation, the embryogenic tissues were subcultured three times on fresh medium and kept in the dark for development of early-stage somatic embryos. The fresh weight of embryogenic tissues was measured at the end of each subculture. For somatic embryo maturation, embryogenic tissues from material derived from each provenance were transferred to medium containing abscisic acid (ABA) to allow maturation of the somatic embryos. The maturation medium was BM1 medium supplemented with 20 μM ABA, 2% sucrose, and 7.5% polyethylene glycol (PEG-4000). Embryogenic tissue clumps growing vigorously and of about the same size (about 1 cm^3 in volume) were selected to monitor the embryo maturation process. The cultures were maintained in the dark at 20°C for about five weeks until the somatic embryos reached cotyledonary stage. Thirty randomly selected mature somatic embryos per provenance were transferred to germination BM1 basal medium supplemented with 0.2% activated carbon (AC), 2% sucrose and 0.4% Phytogel. The embryos were cultured under photoperiod (16h/8h, light/dark) for eight weeks, after which time the plantlets were transferred to *ex vitro* conditions. They were placed in multipot PVC pots filled with a mix of pumice, peat, and organic fertilizer, and they were transferred to greenhouse conditions.

Collection data and statistical analysis

With regard to seedling emergence in the greenhouse, the number of seeds that were sown on each seed bed for each provenance was calculated using the proportion:

Weight in grams of 1000 seeds:1000=Weight in grams of seeds sown on each bed:x

For each provenance, seedling emergences were normalized with respect to the number of seeds put to germinate on each bed. Therefore, the data on the emergence of seedlings from different provenances were normalized to 100 according to this proportion:

Seedling emergence:Total seeds put to germinate x row of seed bed=x:100

Seedling data (emergence and height) were arranged in a completely randomized design, and the statistical significance of all differences in emergence and height of the seedlings was tested using factorial analysis of variance (ANOVA), with the substrate and provenance as the main factors. Differences among provenances were evaluated using Tukey's Multiple-Range Tests (Snedecor and Cochran, 1980) at the 5% level of significance. For seedling growth parameters, means and standard errors were calculated for material derived from each provenance. All statistical analyses were performed with STATISTIX software version 8 (Analytical Software, Tallahassee, FL, USA).

3. Results

In vivo *experiment*

The three provenances significantly ($p<0.05$) varied in emergence and seedling height (cm) in the two different substrates used in the seed beds (Table 3). Seed provenance significantly affected all these processes. In addition, the substrate effect was also significant except at 15 days after sowing, and the substrate x provenance interaction effect was also significant. More precisely, seedling emergence in the greenhouse occurred 15 to 30 days after sowing, and thereafter it was very low or non-existent (data not shown). With regard to substrate 1, which contained a lower organic matter content, seedling emergence differed significantly among the three provenances at both 15 and 30 days after sowing. At 15 days, VF showed 58% seedling emergence, whereas GS and PF showed 38 and 20% seedling emergence, respectively. After 30 days, the percentage of seedling emergence for PF increased to 25%, whereas GS and VF (Fig. 1A) showed the highest seedling emergences of 62 and 91%, respectively. As for substrate 2, which contained the highest organic matter content, the emergence percentage ranged from 34% for GS to 36 and 38% for VF and PF, respectively, after 15 days. Subsequently, a large percentage of seedlings for GS and PF appeared flaccid and twisted as if suffering from wilt. These seedlings continued to decline, assumed a dark colour and greasy appearance, and then quickly disintegrated to leave areas of bare soil in the seed bed (Fig. 1B). After 30 days, seedling emergence was reduced to 20%, whereas that for VF increased to 47%.

Seeds from the VF provenance generated the most seedlings on both substrates; seeds from PF displayed the worst performance on the same substrates at the end of culture in the greenhouse (Table 3). In contrast, GS provenance produced the most seedlings only on substrate 1. The final stem height was determined for all seedlings at 90 days from sowing. The stem height of seedlings ranged from 2.4 to 4.3 cm on substrate 1, and from 2.1 to 3.2 cm on substrate 2. VF seedlings showed the greatest stem height on substrate 1; PF seedlings exhibited the lowest on both substrates. The stem height of GS seedlings was highest on substrate 1.

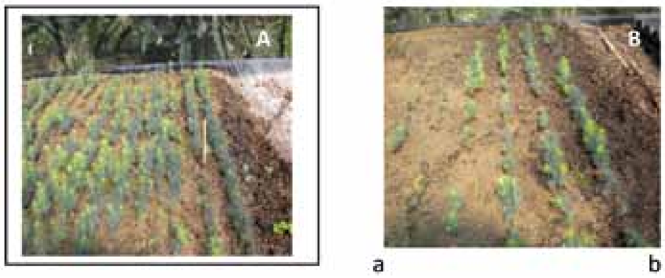

Fig. 1 - Emergence and growth of seedlings on different seed beds at 30 days after sowing: (A) seedlings from the provenance Val di Fiemme on substrate 1; (B) at left, seedlings from the provenance Pezzel and Fochino, showing areas of bare soil, and at right, seedlings from the provenance Val di Fiemme on substrate 2.

The growth trends were not different for seedlings of the provenances measured after 4, 12 and 52 weeks cultivation in pots (Fig. 2). At the time of initial measurement (4 weeks), PF (7.9 cm) and GS (7.4 cm) seedlings showed superior stem growth compared to VF seedlings (6.4 cm). At the end of the cultivation period (52 weeks), GS and PF seedlings had mean stem heights of 14.7 and 13.8 cm, respectively, whereas VF seedlings attained a mean height of 10 cm (Fig. 2). The minimum standard defined by the European Economic Community for growth of three-year-old seedlings is 14 cm in height (E.E. C. Minimum Standards) (Magini, 1977). Therefore, the recorded growth in seedlings of 15 months old from GS and PF provenance exceeded the minimum standard. Among the provenances, seedlings grown in pots exhibited changes in early growth, and fresh and dry matter allocation (Table 4). GS seedlings showed significantly higher values in fresh shoot biomass. On the other hand, VF provenance, compared with GS,

Table 3 - Seedling emergence and early growth of three provenances of *P. abies* in two different substrates

Growing medium	Provenance	Seedling emergence (%)		Seedling height [z] (cm)
		15 days	30 days	
Substrate 1	Gran Bosco di Salbertrand	38 b	62 b	4.3 a
	Pezzel e Fochino	20 c	25 cd	2.4 d
	Val di Fiemme	58 a	91 a	3.7 b
Substrate 2	Gran Bosco di Salbertrand	34 bc	20 d	2.6 d
	Pezzel e Fochino	38 b	20 d	2.1 d
	Val di Fiemme	36 bc	47 bc	3.2 c
Two-way ANOVA (P values)				
Substrate (S)		0.4152	0.0000	0.0000
Provenance (P)		0.0005	0.0000	0.0000
S x P		0.0001	0.0013	0.0000

Values for each parameter, column, and factor followed by different letters are significantly different (Tukey's multiple range test, $p < 0.05$).
[z] The final stem height was determined for all seedlings at 90 days from sowing: the average for Gran Bosco di Salbertrand, Pezzel e Fochino and Val di Fiemme was calculated on 200, 123 and 331 seedlings for the substrate 1 and on 867, 257 and 801 seedlings for the substrate 2, respectively.

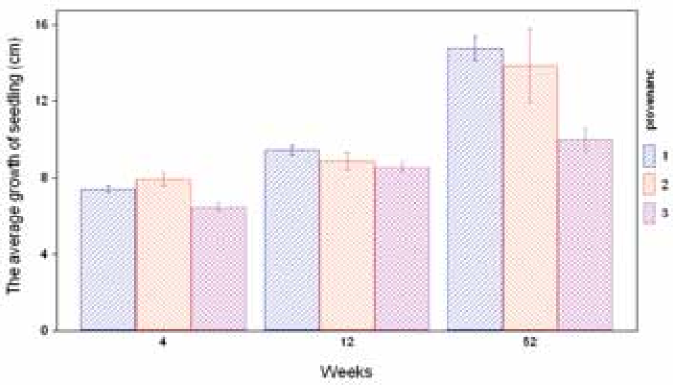

Fig. 2 - Stem heights of *Picea abies* seedlings grown in pots outdoors measured after 4, 12, and 52 weeks of cultivation in pots. Data represent the means (±SE) for seedlings from the Gran Bosco di Salbertrand (1), Pezzel e Fochino (2), and Val di Fiemme (3) provenances. A minimum of 200 seedlings were measured for each provenance.

showed similar values in dry shoot biomass, root biomass, and root/shoot ratio, but lower values were always found in PF provenance.

In vitro *experiment*

Explants from all examined provenances give rise to embryogenic tissues (Table 5). The mature zygotic embryos from GS seeds yielded a higher frequency (13.5%) of embryogenic tissue induction, compared with those for PF (8.6%) and VF (5.5%). A higher frequency (13.4%) of embryogenic tissue induction, independent of provenance, was achieved on medium supplemented with low concentrations of growth regulators (5 µM 2,4-D and 2.5 µM BA) than on medium with higher concentrations of growth regulators (10 µM 2,4-D and 5µ M BA). With regard to the proliferation of embryogenic tissues, only the provenance GS showed the highest increase in fresh weight of embryogenic tissue and the capacity for embryo maturation in the presence of low concentrations of growth regulators. In contrast, a greater increase in embryogenic tissue fresh weight was achieved for all provenances on medium supplemented with the higher concentrations of 2,4-D and BA. These cultures were whitish to translucent, and were characterised by vigorous growth (Fig. 3 A, B); maturation of embryogenic tissues for each provenance is reported in Table 5. Specifically, GS and PF embryogenic tissues showed the highest maturation efficiency (mean of 52 and 73 somatic embryos per clump, respectively), whereas the VF embryogenic tissues produced an average of only 24 somatic embryos per clump. The maturation process up to the cotyledonary stage required about 5 weeks of culture on the maturation medium (Fig. 3 C-E). Over 74% of the somatic embryos germinated rapidly after transfer to the germination medium. The development of plantlets with true needles was observed within 1 to 2 weeks (Fig. 3F).

Table 4 - Morphological characteristics of *Picea abies* seedlings grown in pots outdoors

Provenance	Mean number		Shoot biomass (g)		Root biomass (g)		Root to shoot ratio
	Branche	Roots	Fresh	Dry	Fresh	Dry	
G S	3.0±0.46 a	9.7±1.34 a	5.15±0.09 a	2.71±0.08 a	1.23±0.12 a	1.06±0.07 a	0.395 a
P F	1.3±0.44 a	7.0±1.29 a	2.15±0.18 c	1.88±0.16 b	0.52±0.12 b	0.44±0.07 b	0.257 a
V F	1.8±0.63 a	7.0±1.29 a	4.45±0.10 b	2.66±0.09 a	1.16±0.14 a	1.05±0.09 a	0.390 a

All parameters of growth were evaluated after 52 weeks of growth in pots. The data were subjected to analysis of variance. Values are the mean of a minimum of 15 seedlings for each provenance. Means within a column followed by a different letter are significantly different (Tukey's multiple range test, P< 0.05).
GS= Gran Bosco di Salbertrand;
PF= Pezzel e Fochino;
VF= Val di Fiemme.

Table 5 - Induction of embryogenic cultures, proliferation, and somatic embryo maturation

Provenance	Embryogenic cultures (%)	Increase of mass embryogenic (g)		Embryo maturation	
		5µM 2.4D+ 2.5µM BA	10µM 2.4D+ 5µM BA	No. somatic embryos/clump	Total no. embryos/clump
Gran Bosco Salbertrand	13.5±2.35 a	1.09±0.60 a [(z)]	1.51±0.26 a	52.0±15.0 b	619
Pezzel e Fochino	8.6±2.72 a	0.22±0.15 a	1.66±0.39 a	73.0±11.5 a	881
Val di Fiemme	5.5±2.11 a	0.30±0.10 a	1.13±0.48 a	24.0±9.0 c	288
Growth regulators					
5µM 2.4D+ 2.5µM BA	13.4±1.57 a				
10µM 2.4D+ 5µM BA	5.0±2.72 b				

[(z)] Data represent increase of fresh weight over time. The data were subjected to analysis of variance. Values within a column followed by a different letter are significantly different (Tukey's multiple range test, P < 0.05).

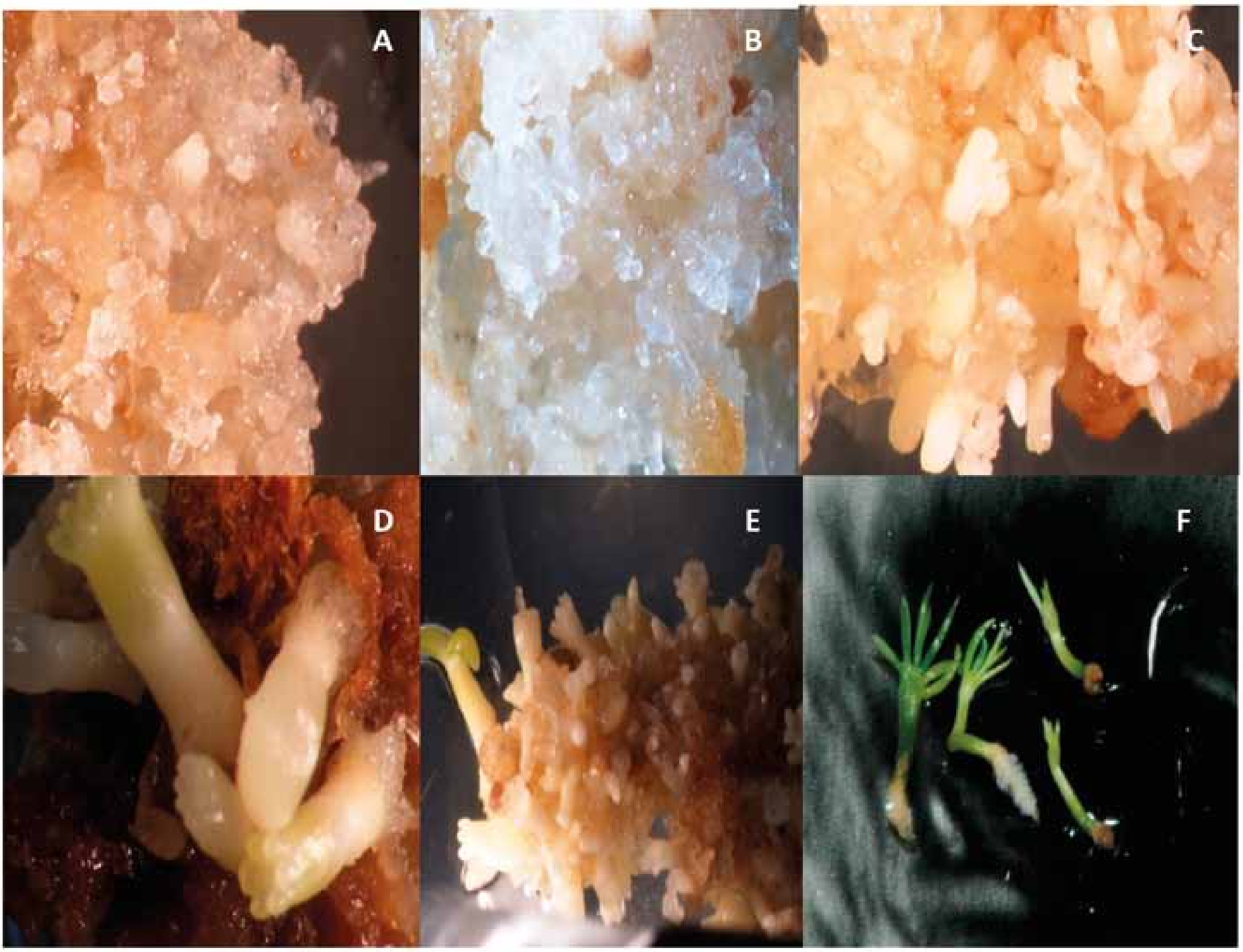

Fig. 3 - Somatic embryogenesis from mature zygotic embryos of *Picea abies*; proliferation of embryogenic tissues (A and B); maturation (C-E) and germination (F) of somatic embryos.

4. Discussion and Conclusions

The efficiency of *P. abies* propagation from seeds and by somatic embryogenesis was closely related to the provenance of the plant material. Furthermore, the emergence and growth of seedlings propagated in seed beds, and the capacity for somatic embryogenesis were influenced by the composition of the growing medium. With regard to seed propagation, the best performance in terms of production and seedling growth was achieved by the Val di Fiemme provenance on both growing media, whereas the Pezzel e Fochino provenance showed the worst performance. Moreover, the positive results of VF provenance on emergence and growth of seedlings in seed beds are also confirmed by the best values regarding seed quality of this provenance. In addition, the organic matter added to growth medium and its content should be taken into account. Malakouti and Homaei (2004) reported that organic matter improved seed germination and seedling growth, providing suitable conditions for seedling production. On the contrary, this study shows that the highest content of organic matter seems to induce a detrimental effect on growth and seedling survival. Probably high amounts of organic matter in the nursery environment promotes favourable conditions for the development of pathogens living in the growing medium. Therefore, the mortality of Gran Bosco di Salbertrand and Pezzel e Fochino seedlings could be due to a disease, likely damping off, as evidenced from areas of bare soil in the seed beds. In contrast, the VF seedlings showed superior growth and survival and seem to be more resistant to pathogens. Consequently, control of organic-matter content, humidity level in seed beds, and reduction in the duration of cultivation under greenhouse conditions are important factors to achieve a high percentage of seedling emergence and growth. At the end of the cultivation period in pots, the fifteen-month seedlings of GS provenance showed a height growth in excess of the minimum standard for three-year-old seedlings. However, the VF and GS provenances showed similar values in dry matter, while lower values were always found in PF. On basis of these results, the production of Christmas trees could be increased by the use of Val di Fiemme seeds, which showed higher germinability and growth of seedlings and by shortening the time for transplanting.

To the authors' knowledge, this is the first report of the application of somatic embryogenesis technology to *P. abies* seeds from different Italian provenances. Our results demonstrate a low production of embryogenic tissues, and among the provenances a different efficiency was evident with regard to the proliferation and maturation of somatic embryos. Only the GS provenance was able to develop somatic embryos capable of germination on both proliferation media; the other provenances gave rise only to somatic embryos on medium with a high concentration of growth regulators. Specifically, GS and PF provenances showed the highest maturation efficiency in terms of the number of embryos per embryogenic clump. These results obtained *in vitro* are in line with previous studies conducted on conifers. Chen *et al*. (2010) reported low frequency of embryo initiation and genetic specificity of explants as serious problems associated with embryogenesis induction. Moreover, the induction of somatic embryogenesis varies greatly among different genotypes under identical culture protocols and proliferation and maturation levels have been identified as major constraints to somatic embryogenesis (Tang *et al*., 2001).

In order to improve the efficiency of propagation from seeds and by somatic embryogenesis, further studies will be finalized to manipulate the composition of the growing medium to improve the emergence and growth of seedlings and frequency of embryogenic tissues.

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