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Seed germination after freezing in high-mountain plant species: Implications for ski-run restoration

Germinación de semillas después de su congelamiento en plantas de alta montaña: Implicaciones para la restauración de pistas de esquí

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Abstract. The construction of a ski slope implies a strong environmental impact as a result of the removal of the vegetation cover. The need to protect the soil requires a rapid restoration of vegetation, which is often done with commercial seed mixtures that can cause a negative impact on these high mountain ecosystems. Thus, the use of seeds of native species is essential, especially in areas rich in endemic species. The compaction of snow as a result of the preparation of the ski slopes causes the soil to freeze. This hinders the germination of seeds, especially those of shrub species. This paper analyzes, under laboratory conditions, how freezing and phytohormone application affect the seed germination of three endemic shrubby species used in the restoration of the ski slopes on Sierra Nevada (S. Spain). Freezing considerably reduced the germination of all species. However, when seeds were previously subjected to phytohormones, the germination percentage increased up to 10-fold with respect to seeds not treated with growth regulators. The results suggest that treating seeds with phytohormones could improve the restoration of the ski slopes with native species.

Keywords: Germination; High-mountain plant species; Plant-growth regulators; Ski-slope restoration; Soil freezing.

Resumen. La construcción de una pista de esquí implica un fuerte impacto como consecuencia de la eliminación de la cubierta vegetal. La necesidad de proteger el suelo requiere una rápida restauración de la vegetación lo cual se lleva a cabo muchas veces con mezclas comerciales de semillas, lo que puede causar un impacto negativo sobre estos ecosistemas de alta montaña. Así, la utilización de semillas de especies nativas es esencial, especialmente en áreas ricas en especies endémicas. La compactación de la nieve como consecuencia de la preparación de las pistas de esquí tiene como resultado la congelación del suelo, lo que puede afectar a la germinación de semillas, sobre todo a las de especies arbustivas. En este trabajo, realizado en laboratorio, se analiza el efecto de la congelación y la aplicación de fitohormonas en la germinación de semillas de tres especies arbustivas y endémicas utilizadas en la restauración de las pistas de esquí de Sierra Nevada (Sur de España). La congelación redujo considerablemente la germinación de todas las especies estudiadas. No obstante, si las semillas fueron previamente tratadas con reguladores del crecimiento vegetal, la germinación se incrementó hasta diez veces con relación a la germinación de las semillas no tratadas con reguladores del crecimiento. Los resultados sugieren que estos tratamientos podrían ser una importante herramienta para promover la restauración de las pistas de esquí con especies nativas.

Palabras clave: Congelación del suelo; Especies de alta montaña; Germinación; Reguladores del crecimiento vegetal; Restauración de pistas de esquí.

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INTRODUCTION

High-mountain ecosystems are subjected to human exploitation through outdoor recreational activities (Körner, 2002). Ski resorts is one of the major economic activities in these areas (Elasser & Masserli, 2001; Isselin-Nondedeu & Bédécarrats, 2007). Ski-run construction and summer maintenance cause severe disturbances especially in the graded ski slopes, which are cleared and then machine-leveled to remove rocks and slope irregularities (Burt & Rice, 2009). This disturbance removes the upper soil layers and the vegetation (Ruth-Balaganskaya & Myllynem-Malinem, 2000; Rixen et al., 2004a; Wipf et al., 2005; Pickering & Hill, 2007). All this disruption makes the slopes more prone to the loss of soil, seeds, nutrients, and organic matter when intense rainfall occurs (Isselin-Nondedeu & Bédécarrats, 2007; Zuazo & Pleguezuelo, 2008), a fact that is aggravated given that alpine ecosystems are particularly fragile (Clifford, 2002; Rivera & Leon, 2004). In addition, these are often singular areas from a conservation standpoint, harboring a high rate of endemic, endangered or protected species (Korner, 2002; Väre et al., 2003). As a result, the interest and effort to restore the natural vegetation damaged by previous and present outdoor recreational activities in alpine areas has grown in recent decades (Chambers, 1997; Muller et al., 1998).

The restoration of plant cover in disturbed alpine ecosystems presents great difficulties given various factors such as low temperatures, high radiation levels, wind, and irregular topography (Bayfield, 1996; Titus & Tsuyuzaki, 1999). In the case of the ski slopes, ski-run preparation and the skiers themselves compact the snow, increasing its density. This implies greater thermal conductivity, which in turn provokes a sharp soil-temperature drop even to levels below 0 °C. The main consequence is the induction of soil frost (Rixen et al., 2003; Rixen et al., 2004b). As a result, plant species with insufficient cold resistance may be damaged, resulting in a higher proportion of unvegetated ground (Wipf et al., 2005).

Disturbed ski slopes are re-vegetated to establish a stable vegetation cover as quickly as possible and to protect the soil against erosion (Van Ommeren, 2001; Krautzer et al., 2006). However, re-vegetation of bare soil is difficult and slow (Barni et al., 2007). Moreover, there is a general lack of knowledge about the conditions needed to promote the germination of native species in alpine ecosystems (Urbanska, 1997; Lorite et al., 2007). Because of this, commercial, non-native species are commonly used for the regeneration of plant cover in ski runs (Kangas et al., 2009; Burt, 2012). However, with this practice, exotic species can invade communities of native and endemic species (Thompson, 2005). This occurs at the Sierra Nevada ski resort (SE Spain), an area surrounded by a National Park that harbors ca. 80 endemic plant taxa exclusive to these cacuminal areas (Blanca, 2002), and where ski-slope restoration efforts have not achieved their desired results (Lorite et al. 2010).

The maintenance of a sustainable plant community in the ski runs is needed to ensure optimal ecosystem functioning (Mulder et al., 2001; Wipf et al., 2005). Thus, it is necessary to find methods that enable the restoration of the vegetation cover of the ski slopes with native plants (Burt, 2012). Therefore, knowledge is essential concerning the different factors that control the germination process in these wild plants, which often go into dormancy (Bewley & Black, 1994; Baskin & Baskin, 1998). Among other factors, such as light, temperature, scarification, etc., phytohormones are presumed to have a determining role in this process (Kucera et al., 2005). However, no studies are available on how seeds, after being frozen, respond to treatment with plant growth regulators. This is a critical point to consider for the restoration of ski-runs via seed sowing, as it is common that seeds undergo frozen temperatures below the snow cover due to the snow compaction in the period that spans from sowing (summer or autumn) to seed germination (the following spring).

In the present study, we analyze the effects of certain plant-growth regulators and freezing on the germination of three endemic, alpine plant species common in the ski area of Sierra Nevada, Spain. We hypothesize that the use of phytoregulators may promote the germination of these species since they have low germination rates (Lorite et al., 2007). Given that natural conditions after sowing on ski runs imply freezing temperatures, we checked for the effect of freezing after the hormonal treatments. This allows the simulation of sowing conditions under ski-run management. Overall, we seek to study whether freezing after hormone application might be a suitable approach to promote germination. This might help restore the ski slopes using native vegetation and, hence, promote the conservation of native vegetation in these disturbed areas.

MATERIALS AND METHODS

Study species and seeds collection. Seeds of *Genista versicolor* Boiss (Leguminosae), *Reseda complicata* Bory (Resedaceae) and *Thymus serpylloides* Bory subsp. *serpylloides* (Labiatae) were used for the study. The three species are endemic of the Baetic range and grow above 2000 m.a.s.l. (Table 1). They are perennial chamaephytes (shrubs or scrubs) common in the area occupied by the Sierra Nevada ski resort. The three species are used in the restoration of the ski runs of Sierra Nevada, representing over 22% of the species used (Lorite et al., 2010). Seeds of the three species were collected from natural populations at coordinates 37° 05' N and 3° 23' W and at an altitude of 2500 m. Seeds were collected from 30 mother plants per species (12 plants in the case of *R. complicata*), and afterward they were pooled for each species. After collection, the seeds were extracted manually from the fruits under laboratory conditions and sorted with the use of a magnifying glass, discarding damaged ones (those with clear signals of injury

Table 1. Summary information about the species used in this study. Distribution code: BM = Endemic Baetic Mountains, VU = Vulnerable.
Tabla 1. Resumen sobre las especies utilizadas en este estudio. Códigos de distribución: BM = Endemismo de las Montañas Béticos, VU = Vulnerable.

Species	Family	Distribution	Habitat	Altitudinal range	Conservation status
Reseda complicata	Resedaceae	Endemic BM	Montane cushion scrubs	2000-3400	VU
Thymus serpylloides	Labiatae	Endemic BM	Montane cushion scrubs	2000-3400	__
Genista versicolor	Leguminosae	Endemic BM	Montane cushion scrubs	1600-2600	__

in the cover, or clearly empty). The seeds were then stored for three months at 4 °C.

Sierra Nevada (SE Spain) has the southernmost ski resort in Europe. Temperatures can reach a low of -14 °C in the coldest month and a high of 26 °C in the warmest month (measured in a meteorological station placed in the ski resort at 2800 m.a.s.l.). The average annual precipitation is 925 mm, mainly in the form of snow, reaching a thickness of more than 2 m in the ski resort. However, in some areas, 5 m of thickness can be reached (Gómez, 2002).

Experiment 1. Effect of phytoregulators on seed germination. In the first experiment, seeds were sterilized superficially with sodium hypochlorite at 1% for ten minutes and washed thoroughly with distilled, sterilized water. Subsequently, they were subjected to seven treatments with different plant growth regulators and concentrations: 1: Control (C); 2: Bencylaminopurine (BAP) at 10^{-6}M concentration; 3: Bencylaminopurine at 10^{-5}M; 4: Gibberellic acid type 3 (GA_3) at 10^{-6}M; 5: Gibberellic acid type 3 at 10^{-5}M; 6: Ethrel at 10^{-6}M; and 7: Ethrel at 10^{-5}M. In all cases, seeds were imbibed in the phytorregulator concentration for 6 h (in distilled water for the Control treatment). Once this time span was complete, seeds were then sown on 9 cm diameter Petri dishes, on two discs of filter paper Whatman grade 1 resting in 6 mm glass pearls placed on the bottom. Afterwards each Petri dish was watered with 20 mL of distilled, sterile water. We used four replicates per experimental treatment (four Petri dishes), each containing 50 seeds. Following this, the dishes were moved to a germination chamber with temperatures of 25/5 °C (light/dark, 14/10 hours, respectively) and a relative humidity of 70%. In the light, the seeds were exposed to cool-white fluorescent light (PAR, 20 μmol/m²/s at seed level). These are conditions similar to those existing on the Sierra Nevada at the end of spring when the seeds germinate in their natural conditions. The germination was measured daily during a 20-day period and a seed was considered to have germinated when the radicle pierced the cover.

Experiment 2. Effect of freezing on seed germination. The effect of freezing in seed germination was analyzed exposing seeds to the same treatments used in Experiment #1. For this, subsamples of seeds from the Control treatment and all the growth regulators treatments were placed in vials (one per treatment), and then stored at -20 °C for three months. After this time, the seeds were sown in the same way and under the same conditions as in the previous experiment. Similarly, sample size was 4 Petri dishes per treatment, each containing 50 seeds (thus 200 seeds per species in total).

Seed water imbibition. The fresh weight of seeds was determined for each species weighing four samples of 50 seeds each in the case of *G. versicolor*, and 100 seeds each in the other two species. Subsequently, the seeds were imbibed in water for 6 hours and weighed to determine the amount of water absorbed after that time; previous trials showed that imbibition reached its maximum value after this time. The dry weight of each sample was determined by drying the seeds in an oven at 105 °C for 24 hours.

Data analysis. The cumulative seed germination for non-frozen seeds was analyzed with one-way ANOVAs for each species. The germination of seeds frozen after phytoregulators application was analyzed by two complementary ways. First, we used a failure-time approach using a Weibull distribution, which measures the time to failure of each individual seed (Fox, 1993). In addition, cumulative germination after 20 days of experimentation was compared among treatments and for every independent species with a one-way ANOVA to explore the final result without the influence of the shape of the survival curve. Post-hoc comparisons after ANOVAs were performed with the Duncan test at a p-value of 0.05. For ANOVAs, the data were arcsin transformed to improve normality and homoscedasticity.

RESULTS

The results of the first experiment, with unfrozen seeds, showed that at least some of the concentrations that were applied improved germination. For *R. complicata* the highest germination rate resulted with BAP 10^{-5}M (up to 75% germination with BAP vs. 40% for control seeds), followed by GA_3 10^{-5}M and Ethrel 10^{-6}M (all of them with significant differences respect to the Control; Table 2). For *T. serpylloides*, the highest germination rate was reached with GA_3 and BAP, irrespective of the concentration (2-fold the germination reached by control

Table 2. Summary of the germination percentages for non-frozen seeds after different phytoregulator applications (treatments). Values are means ± standard error after 20 days of experiment. Treatments were: Control (water), Bencylaminopurine at 10^{-6}M (B6), Bencylaminopurine 10^{-5}M (B5), Ethrel 10^{-6}M (E6), Ethrel 10^{-5}M (E5), Gibberellic acid 10^{-6}M (G6) and Gibberellic acid 10^{-5}M (G5). In all cases, seeds were imbibed in the treatment concentrations for 6 h. Degrees of freedom were 6 (treatment) and 21 (error) in all cases. Different letters show statically differences among treatments for each species according to the Duncan test at a p-value of 0.005.

Tabla 2. Resumen sobre los porcentajes de germinación de semillas sin congelar después de la aplicación de diferentes fitorreguladores (tratamientos). Los valores expresan las medias ± error estándar después de 20 días de experimento. Los tratamientos fueron: Control (agua), Bencylaminopurina a 10^{-6}M (B6), Bencylaminopurina 10^{-5}M (B5), Ethrel 10^{-6}M (E6), Ethrel 10^{-5}M (E5), ácido Giberélico 10^{-6}M (G6) y ácido Giberelico 10^{-5}M (G5). En todos los casos, las semillas fueron imbibidas en las diferentes soluciones durante 6 horas. Los grados de libertad fueron 6 (tratamientos) y 21 (error) en todos los casos. Letras diferentes muestran diferencias estadísticamente significativas entre tratamientos para cada especie de acuerdo con el test de Duncan (p-valor de 0,005).

Species	Treatments							F	P
	Control	BAP 10^{-6}M	BAP 10^{-5}M	Ethrel 10^{-6}M	Ethrel 10^{-5}M	GA3 10^{-6}M	GA3 10^{-5}M		
Reseda complicata	40.5 ± 1.7a	38.0 ± 4.9a	74.5 ± 2.5b	43.5 ± 3.0ac	51.0 ± 1.3c	42.5 ± 3.8ac	60.0 ± 0.8d	20.26	<0.0001
Thymus serpylloides	24.5 ± 2.6a	48.0 ± 3.7b	48.0 ± 2.6b	31.0 ± 1.0a	30.0 ± 1.6ª	50.0 ± 2.0b	51.5 ± 1.3b	24.31	<0.0001
Genista versicolor	33.0 ± 1.3a	58.0 ± 4.2bc	52.0 ± 6.9bc	56.5 ± 2.8c	62.0 ± 2.9c	48.0 ± 1.8b	51.5 ± 3.8bc	6.08	0.0008

Table 3. Summary of the results of the survival analysis for germination of seeds subjected to freezing at -20 °C after phytoregulator application. Survival analysis was done fitting the data to a Weibull distribution. See Figures 1A, B and C for germination curves across the days.

Tabla 3. Resumen de los resultados del análisis de supervivencia para la germinación de semillas sometidas a congelación a -20 °C después de la aplicación de fitorreguladores. El análisis de supervivencia fue hecho adecuando los datos a una distribución Weibull. Ver Figuras 1A, B y C, para las curvas de germinación a lo largo del tiempo.

Species	L-R Chi Square	Df	P
Reseda complicata	426.91	6	<0.0001
Thymus serpylloides	52.69	6	<0.0001
Genista versicolor	8.29	6	0.2178

Table 4. Seed mass for 100 seeds expressed in mg. FW: fresh weight; DW: dry weight; MCDW: moisture content in % on dry weight after 6 hours of imbibitions. Values are mean ± SE of 4 replicates.

Tabla 4. Peso de 100 semillas expresado en mg. FW: peso fresco; DW: peso seco; MCDW: contenido en agua % sobre peso seco después de 6 horas de imbibición. Los valores son la media ± SE de 4 réplicas.

Species	FW 100 seeds (mg)	DW 100 seeds (mg)	% MCDW
Reseda complicata	9.1 ± 0.3	8.2 ± 0.3	22.4 ± 0
Thymus serpylloides	22.3 ± 2.3	17.8 ± 1.8	54.4 ± 2
Genista versicolor	355.2 ± 32	318.3 ± 3	53.3 ± 6

seeds). For *G. versicolor*, all treatments improved germination, which also reached values close to 2-fold the values of control seeds (up to 62% with Ethrel *vs* 33% for Control; Table 2).

For seeds subjected to freezing (Experiment 2), germination also differed among treatments for two of the species, *R. complicata* and *T. serpylloides* (Table 3). For *R. complicata*, germination reached the maximum values with BAP 10^{-5}M and GA_3 10^{-6}M (about 50%), and the minimum with the control treatment (5%; Fig. 1A). For *T. serpylloides*, the maximum germination was reached with BAP 10^{-6}M (20%) and the lowest with Ethrel 10^{-6}M and control (3%; Fig. 1B). Finally, *G. versicolor* showed no significant differences among treatments, despite that the highest values were also reached with phytoregulator application (Ethrel 10^{-6}M; Fig. 1C).

The seeds of all the species absorbed water after the six h of imbibitions, with values that ranged between 22.4% of weight for *R. complicata* to 54.4% for *T. serpylloides* (Table 4).

DISCUSSION

The possibility that seed germination of the native species can be improved is of great importance in the restoration of ski slopes, since these areas are usually very sensitive to disturbances (e.g. erosion). These areas harbor a high rate of endemic species, and germination in these habitats is often low and poorly understood (Körner, 1999). In the case of the Sierra Nevada ski resort (as it is common in other ski resorts),

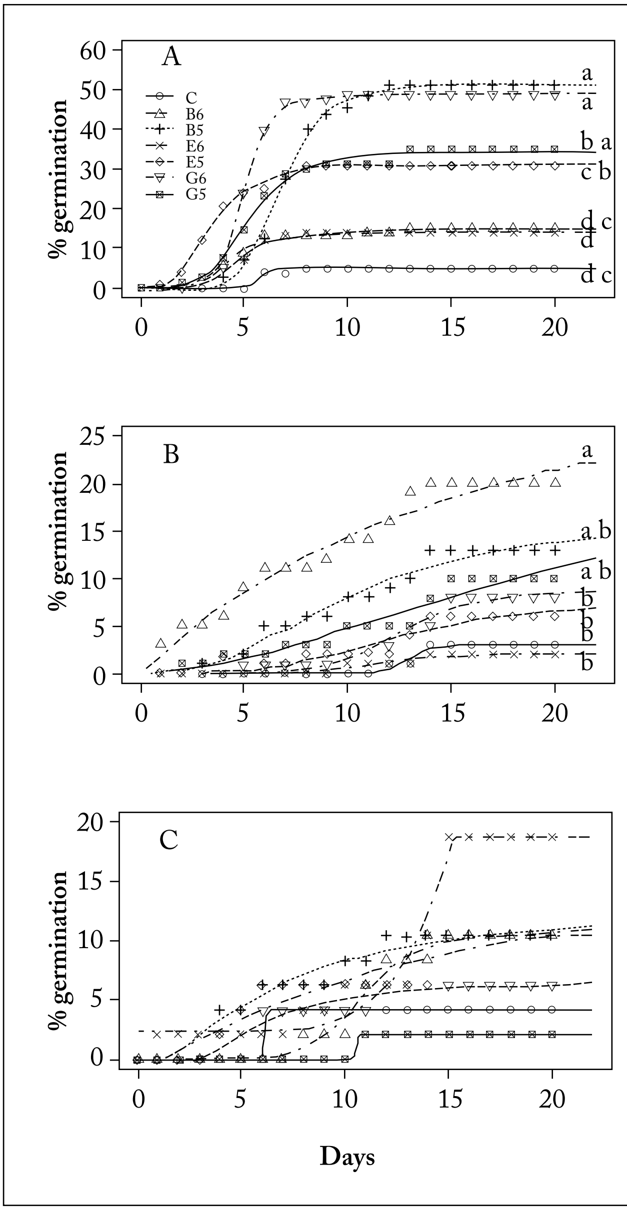

Fig. 1. Germination trends of *Reseda complicata* (A), *Thymus serpylloides* (B) and *Genista versicolor* (C) seeds previously pretreated with Benzyladenine 10^{-6}M (B6), Benzyladenine 10^{-5}M (B5), Gibberellic acid 10^{-6}M (G6), Gibberellic acid 10^{-5}M (G5), Ethrel 10^{-6}M (E6) and Ethrel 10^{-5}M (E5). Subsequently the seeds were frozen to -20 °C for three months. Values are means of four replicates. Different letters at the end of the curves show significant differences (p=0.005).

Fig. 1. Curvas de tendencia de la germinación de semillas de *Reseda complicata* (A), *Thymus serpylloides* (B) y *Genista versicolor* (C) previamente pretratadas con Benzyladenine 10^{-6}M (B6), Benzyladenine 10^{-5}M (B5), Gibberellic acid 10^{-6}M (G6), Gibberellic acid 10^{-5}M (G5), Ethrel 10^{-6}M (E6) y Ethrel 10^{-5}M (E5). Posteriormente las semillas fueron congeladas a -20 °C durante tres meses. Los valores son la media de cuatro réplicas. Letras diferentes al final de cada curva muestran diferencias significativas (p=0,005).

hydroseeding procedures are undertaken in autumn in order to break seed dormancy (Körner, 1999). Thus, in the spring, with higher temperatures and abundant thaw water, the seeds can germinate. However, seedling-recruitment rates, especially among shrub species, are very low (Lorite et al., 2010). A potential cause could be the low soil temperatures as a consequence of snow compaction. The use of plant-growth regulators could help break dormancy and increase the germination.

Our results support the possibility of increasing the germination of native species inhabiting the slopes using three different plant-growth regulators: BAP, GA_3 and Ethrel (a compound that is hydrolyzed and releases ethylene at pH values above 4.0). In the first experiment, the results for the three species studied proved similar to those found in other experiments with the same species. In these experiments, seeds were maintained in solutions with the same plant-growth regulators (Díaz-Miguel et al., 2007; 2011). The effect of freezing on the seed viability and germination in many grasses is not always significant, but it appears to affect woody perennials (Wesche et al., 2006). Regarding the results of germination in the Control treatments, other authors have found lower germination rates at low temperatures (Milbau et al., 2009), as is the case with the data presented here. Our data on no seed germination under a high degree of moisture at -20 °C agree with the results of Kumar & Bahatla (2006).

The effect of hormonal treatments of seeds after a freeze period, a situation common for seeds sown in the ski runs in winter, is largely unknown. The pretreatments used in these experiments were still effective after the seeds were frozen for a certain time period, a result that has not been reported previously. Our results provide therefore novel insights into the use of hormone applications and freeze to promote seed germination in alpine species. Although the germination of the three species was higher before freeze application, the key point is that, after being frozen, the seeds subjected to some growth regulators improved their germination up to 10- 5- and 4-fold in *R. complicata, T. serpylloides,* and *G. versicolor*, respectively (Fig. 1). Given that freezing is normal in ski-run soils, this phytoregulator application may help ensure the success of restoration of these areas. The fact that the highest germination after seed freezing was obtained for the species with lower water imbibition (*R. complicata*) further suggests that seeds with higher water content have a higher probability to form ice crystals that may result in freeze damage (Keefe & Moore, 1983; Bai et al., 1998). For seeds of *R. complicata*, the lowest water content relative to the dry weight could be considered as unfreezable levels (Burke et al., 1976).

In conclusion, the results of this study show that the application of plant-growth regulators may promote seed germination (respect to control seeds) once seeds have suffered a freezing process. This could help to restore plant cover in ski-resort areas, and even contribute to the conservation of endemic and threatened taxa of high mountain ecosystems by improving the restoration success with local, native species.

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