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Seedling establishment of two shrubby plants native to the Sierra Nevada mountain range

Research Article

Francisco A. Serrano-Bernardo[1*], José L. Rosúa-Campos[2]

[1] *Department of Botany, Faculty of Sciences*
University of Granada Campus Fuentenueva,
18071 Granada, Spain

[2] *Department of Civil Engineering, Higher Technical School of Civil Engineers*
University of Granada Campus Fuentenueva,
18071 Granada, Spain

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Abstract: The Sierra Nevada mountain range near the Mediterranean Sea is an unique environment known for the variety of endemic species. Nevertheless, an alpine ski station situated on the mountain has dramatically affected the landscape, leaving some areas barren. In an effort to restore the vegetation cover, laboratory experiments were conducted with seeds of *Genista versicolor* Boiss and *Reseda complicata* Bory, two shrubby plants native to Sierra Nevada. Using different concentrations of two plant growth regulators, Ethrel and N^6-benzyladenine, seeds from both species were planted in soil sampled from the alpine ski resort. Surprisingly, both Ethrel and N^6-benzyladenine significantly improved seedling establishment. Consequently, seedling pre-treatment with definite plant growth regulators could be a useful approach to revegetation of the Sierra Nevada mountain range.

Keywords: *Revegetation • Plant growth regulators • Seedling establishment • Sierra Nevada • Alpine ski slopes • Threatened flora*

Abbreviations

ABA- Abscisic Acid
BA - N^6-Benzyladenine
CEC - Cation-Exchange Capacity
Cks - Cytokinins
Et - Ethrel
PGRs - Plant Growth Regulators

1. Introduction

The alpine region alongside the Mediterranean Sea is one of the worlds most important reserves regarding plant diversity [1]. One of these mountain systems, the Sierra Nevada National Park (Spain), is home to more than two thousand vascular plants (among species and subspecies), constituting more than 30% of the floristic richness of peninsular Spain. Of these vascular plants, eighty are endemic to the Sierra Nevada. In the zone of summit, the percentage of endemic taxa rise to 30% while in exclusive ecological niches, such as rocky or gravelly areas, it reaches 80% [2]. In this unique surrounding, stands the southern most European alpine resort, the Sierra Nevada ski station. Operations required to open and maintain the ski runs have significantly affected the ecosystems and have lead to a major increase in erosion and a heavy loss of biodiversity. To counteract these effects, over the last 15 years, different plant-restoration experiments have been conducted. Laboratory experiments on different germinative pre-treatments for some endemic species by the exogenous application of two known plant growth regulators, N^6-Benzyladenine (BA) and Ethrel (Et) (the latter being the commercial name of 2-chloroethylfosfonic acid, the

* *E-mail: fserber@ugr.es*

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PGR	PGR Concentration/ Immersed Time (ppm/h)	Seed germination (%)	
		Genista versicolor Boiss	*Reseda complicata* Bory
Et	*1/12*	32.0±5.4	26.0±3.3
	1/24	38.0±6.1	18.0±5.4
	10/12	**48.0±9.2**	37.0±7.3
	10/24	40.0±2.6	32.0±5.8
	100/12	**60.0±4.5**	**44.0±9.2**
	100/24	42.0±7.5	**40.0±3.7**
BA	*1/12*	**58.0±7.9**	48.0±7.9
	1/24	44.0±6.6	44.0±6.6
	10/12	54.0±4.9	**56.0±5.5**
	10/24	**56.0±4.2**	**52.0±3.6**
	100/12	32.0±3.8	42.0±5.1
	100/24	40.0±4.1	32.0±5.1

Table 1. Seed germination percentage of *G. versicolor* (GV) and *R. complicata* (RC) under different PGRs pre-treatments ($PGR_{ppm\ /\ immersed\ time\ (h)}$) in previous Petri's dishes experiments. Two best percentages of each specie were selected for this study and marked in bold letters and gray frame. Data shown are the mean from three replicates of 250 seeds each ± S.E. The environmental conditions were 25ºC day /10ºC night temperatures and a photoperiod of 12 h.

Et= Ethrel; BA= N⁶-Benzyladenine

compound that at pH>4-5, on penetrating the tissues releases ethylene) have been conducted. Physiologically, ethylene is involved in a number of plant processes throughout vegetative growth and floral development, fruit senescence [3], ripening in climacteric fruits [4], embryogenesis [5], as well as responses to mechanical injuries, pathogen invasion, abiotic stresses and to the auxin application [6]. While many studies highlight the importance of ethylene during seed germination, its precise role is unclear due to contradictory data. In many species, concentrations of ethylene between 0.1 to 200 µl/l are sufficient to stimulate germination [7-13].

Cytokinins (Cks) are also important growth regulators that control cell division and growth in the presence of auxins [14] and retard leaf, stem, and flower senescence [15]. In addition, Cks also help diminish the inhibitory activity of abscisic acid (ABA) and promote seed germination [16-18]. Numerous kinetin derivatives, such as BA or Benzylaminopurine, in which the furfuryl group is replaced by other groups, also stimulate germination [19,20].

In the present work, *Genista versicolor* Boiss (Leguminosae) and *Reseda complicata* Bory (Resedaceae), two shrubby plants native to the Sierra Nevada mountain range were studied to determine whether the exogenous application of Et and BA could optimise germination and growth. Three different soils sampled from this ecological niche were used with the aim of improving future revegetation works in the ski station.

2. Experimental Procedures

2.1. Seed preparation

Seeds from *G. versicolor* and *R. complicata*, sorted for size and similar external characteristics (discarding those with malformations or anomalies), and were kept in darkness for at least 3 months at 4ºC. *G. versicolor* seeds were imbibed in sulphuric acid for 20 min and then vigorously washed with distilled water and then rinsed with sterilized water. The *R. complicata* seeds were surface sterilized by immersion in 1% sodium hypochlorite for 5 min and then washed as mentioned above. Seeds were placed in Petri dishes prepared with filter paper, and the pre-treatments with plant growth regulators were applied as shown in Table 1. A subsample of seeds was tested for viability with 2, 3, 5-triphenyl tetrazolium chloride [21] and a positive staining reaction was obtained in 80–90% of seeds.

2.2. Soil preparation

Three types of soils were gathered from different zones of the ski station. The determining factors for the selection of the substrates were, among others, slope, orientation, altitude, situation on the ski runs, and human activity. Soil samples were placed in 6 forest flats, for 150 wells each (3 wells/species) with a mean volume of approximately 45 cm^3/well (3 x 3 x 5 cm). Each well was filled with sieved (2-mm mesh size) and sterilized soil. Afterwards the soil was irrigated with distilled water to saturation, and each flat received 750 seeds of each

SOIL	GRAIN ANALYSIS									WATER RETENTION (%)	
	Texture (%)			Gravel (%)	USDA (%) Sand Division						
	Sand	Slime	Clay		Very Thick	Thick	Medium	Fine	Very Fine	33 KPa	1500 KPa
S_1	64.9	26.4	8.7	66.2	18.6	14.7	10.5	12.3	8.8	11.29	5.04
S_2	74.0	20.6	5.4	58.3	20.0	17.1	12.7	14.8	9.4	8.21	2.88
S_3	72.9	22.0	5.1	74.4	33.7	15.2	7.5	9.1	7.4	7.16	2.79

SOIL	CHEMICAL AND PHYSICAL-CHEMICAL PARAMETERS											
	TFV	Appar. Dens. (g/cm^3)	Useful Water (mm/cm)	O.M.(%)	Nitrogen (%)	C/N	P(mg/100g)	pH H_2O	pH KCl	E. C. (mS/cm)	$CaCO_3$ eq. (%)	K^+ (mg/100g)
S_1	0.48	1.48	0.93	1.16	0.11	10.32	7.7	7.1	6.5	0.09	1.66	11.0
S_2	0.57	1.41	0.75	1.93	0.10	19.48	11.4	6.1	5.3	0.04	0	2.9
S_3	0.37	1.58	0.69	0.56	0.09	6.38	8.9	6.2	5.3	0.04	0.77	5.4

SOIL	EXCHANGEABLE BASES COMPLEX ($cmol_{(+)}$ Kg^{-1})												
	Ca^{++}	Mg^{++}	Na^+	K^+	Total Bases	CEC	V (%)	Mn	Zn	Cu	Ni	Co	Cr
S_1	3.4	2.0	0.4	0.3	6.1	8.3	72.8	5.77	1.05	0.52	0.24	0.08	N.D.
S_2	1.5	0.7	0.5	0.1	2.8	7.5	37.1	1.18	0.31	0.43	0.29	0.04	N.D.
S_3	2.3	0.9	0.6	0.1	3.9	6.5	60.5	1.16	0.47	0.12	0.12	0.06	N.D.

Table 2. Main characteristics of the three studied soils.

N. D. = Non detected; TFV = Thick Fraction Volume; CEC: Cation-Exchange Capacity; V (%) = Bases Saturation

species (5 seeds/well) pretreated with Et and BA, with conditions explained in Table 1. Finally, the seeds were covered with a thin layer of soil and watered lightly. The flats were placed in a seedling chamber and were covered with black plastic tarp for 5 days after sowing to conserve moisture.

For an additional test (second experiment) the same protocol was followed with different soils. However, once screened and sterilized, 30 ml of soil and quartz sand (mixed 4:1) were deposited in each well. This quantity was moistened with 5 ml of distilled water, and afterwards the seeds of the different species were covered with a small portion of the soil-sand mixture, to which perlite was added to fill the well. Perlite, a natural mineral of which the main component is aluminium silicate (33-35%), is used to loosen soil and improve drainage as well as retain soil moisture. After sowing, the wells were watered again until the perlite grains were saturated. Periodically, all the flats were watered. The flats were kept at 25ºC (day) and 10ºC (night), with a photoperiod light/darkness of 12/12 h. The experiments lasted 60 days, when the emerged seedlings were counted and afterwards uprooted, washed to eliminate any soil adhering to the roots, and dried in an oven at 80ºC for 24 h. Dry weight was recorded in addition to root and shoot measurement. Duncan's multiple range test was used to analyze differences between the samples (significant at $P<0.05$).

3. Results

The analysis of the three soil samples (Table 2), indicates that the three substrates present a loamy-sandy texture (coarse - very coarse) with a low degree of compaction. These characteristics result in low water retention (none of the soils exceeding 6%), and therefore water needs would be greater in the field. Also, slight acidity was observed in Soils 2 and 3, which, in terms of growth, translates as maximum availability of nutrients for the plants. In contrast to this theoretic availability of nutrients, the low moisture retention of these substrates added to the fact of annual snow cover, implies a greater probability of losses in N and K by leaching, a lowering of salinity and a dramatic reduction in the Cation-Exchange Capacity (CEC).

According to Table 2, after soil screening, the greater degree of compaction was found in Soil 1, most likely due to a higher percentage of clay and a lower fraction of sand. Also, Soil 1 presented a higher quantity of available water, CEC, and exchange bases than the rest, which may indicate greater fertility.

The seedling establishment percentages for *G. versicolor* (GV) and *R. complicata* (RC) seeds at 60 days after imbibition are shown in Figure 1. In the initial experiment, for *G. versicolor*, all pre-treatments were greater than control values, and in particular $Et_{10/12}$ concentration resulted in an 34% improvement in the seedling establishment percentage respect to the control in Soil 1 (S_1). In Soil 2 (S_2), pre-treatments percentages were lower than in S_1, except for $BA_{10/24}$, which improved the previous percentage by 12%. As in S_1 and S_2, in Soil 3 (S_3) all pre-treatments were higher than control, and again $Et_{10/12}$ presents the higher percentage of all.

Among all the treatments applied to the *R. complicata* (RC) seeds sown in S_1, interestingly the seedling establishment percentage for $Et_{100/24}$ was exceeded the control by almost 30%. In addition to this treatment, BA also surpassed control values. In S_3, seeds pretreated with $Et_{100/12}$ presented improved performance, with 80% seedling establishment.

In second experiment, the results for *G. versicolor* (Figure 1) sown in Soil 1 showed an increase in seedling establishment for all treatments in addition to the control. With respect to seedling growth as determined by dry weight (Figure 2), both root and shoot growth increased with the application of $Et_{10/12}$. In Soil 2, the different treatments applied to the *G. versicolor* seeds, as in control, presented seedling establishment percentages higher than those reached in Soil 1. $Et_{100/12}$ in particular showed greater growth, both of the shoot and the root. Moreover, all the treatments presented a shoot/root relationship higher than control. In Soil 3, it was noteworthy that the four treatments with PGRs improved control growth values, both in the shoot and root. Interestingly, the dry weight values recorded for the two Et pre-treatments, showed a reduction in shoot/root indices than BA pre-treatments. In this case, the *R. complicata* seeds (Figure 1) showed a similar trend, followed a set of treatments applied in Soil 1, to increase the seedling establishment percentage in comparison to the first test. Among the growth regulators, the percentages reached by the two Et treatments proved more representative. In terms of shoot growth as inferred from calculations of dry weight (Figure 2), pre-treatment with $BA_{10/12}$ quadrupled in shoot weight when compared to the control seedlings. However, although the percentage of seedling establishment for seeds pretreated with Et was high, the growth of the seedlings was not comparable with the levels recorded for BA treatments.

The results for the *R. complicata* showed that seeds germinated on Soil 2 (Figure 1) had greater seedling viability than Soil 1; however except for treatment with $BA_{10/24}$, the other samples registered notably higher seedling establishment percentages. Remarkable differences appear in relationship between shoot and root dry weight was observed between the entire set of treatments. The seedling establishment data for the *R. complicata* seeds sown on Soil 3 showed a similar trend to Soil 1 and Soil 2 whereby the highest seedling percentages were observed with Et treatments. Furthermore, based on dry weight (Figure 2), seedlings were more developed both in the shoot and in the root, when treated with $Et_{100/12}$. In addition, all the growth regulators tested showed a higher shoot/root relationship than in control.

The analysis of growth, expressed for both the shoot and root as a function of length (Figure 3) showed that BA treatments gave, in general, greater stem lengths, while the shoot growth was shorter on Et application. Also, the four PGRs treatments clearly stimulated root growth in this species respect the control. The effects of the compaction of the substrate are clear, since it is possible to be observed how in both species, the growth, as much of the stem as in the root lengths is larger in the second (d, e, f) than the first experiment (a, b, c).

4. Discussion

Characteristation of the three substrates varied considerably in the laboratory, especially as the soils were filtered with a grid size of < 2 mm that eliminated the coarse fraction. Consequently, a greater amount of fine material would be present resulting in greater compaction. In this sense, the effects of soil compaction on plant growth are complex and can be either beneficial or detrimental. Slight soil compaction after sowing can benefit subsequent growth, as it improves the capillary action of the water towards these seeds. Nevertheless, compaction of the soil is predominantly deleterious as it increases inhibition rates of germination and growth [22]. The degree of soil compaction depends on many factors including texture, pH, CEC, coarseness of the clay particles, water content, and the presence of organic matter, iron oxides, or free aluminium hydroxide [23]. The most important physical aspects of the soil is the lowering of quantities of available O_2 and the rise in those of CO_2 [24], the decline in the infiltration rates, permeability, irrigation [25], the formation of surfaces crusts and increased runoff and erosion [26], with the consequent loss in nutrients [27]. Also, the absence or reduction in organic matter content can have implications for soil formation, and thus seedling growth as it alters water movement and storage, cation exchange, and nutrient supply [28]. Nevertheless, the greater or lesser degree of compaction in these substrates can, to different degrees, degrade soil fertility, deteriorating the physical properties that directly affect plant growth: available H_2O, O_2 supply, temperature, or mechanical resistance. All seedling establishment percentages were higher when quartz sand and perlite was added to the

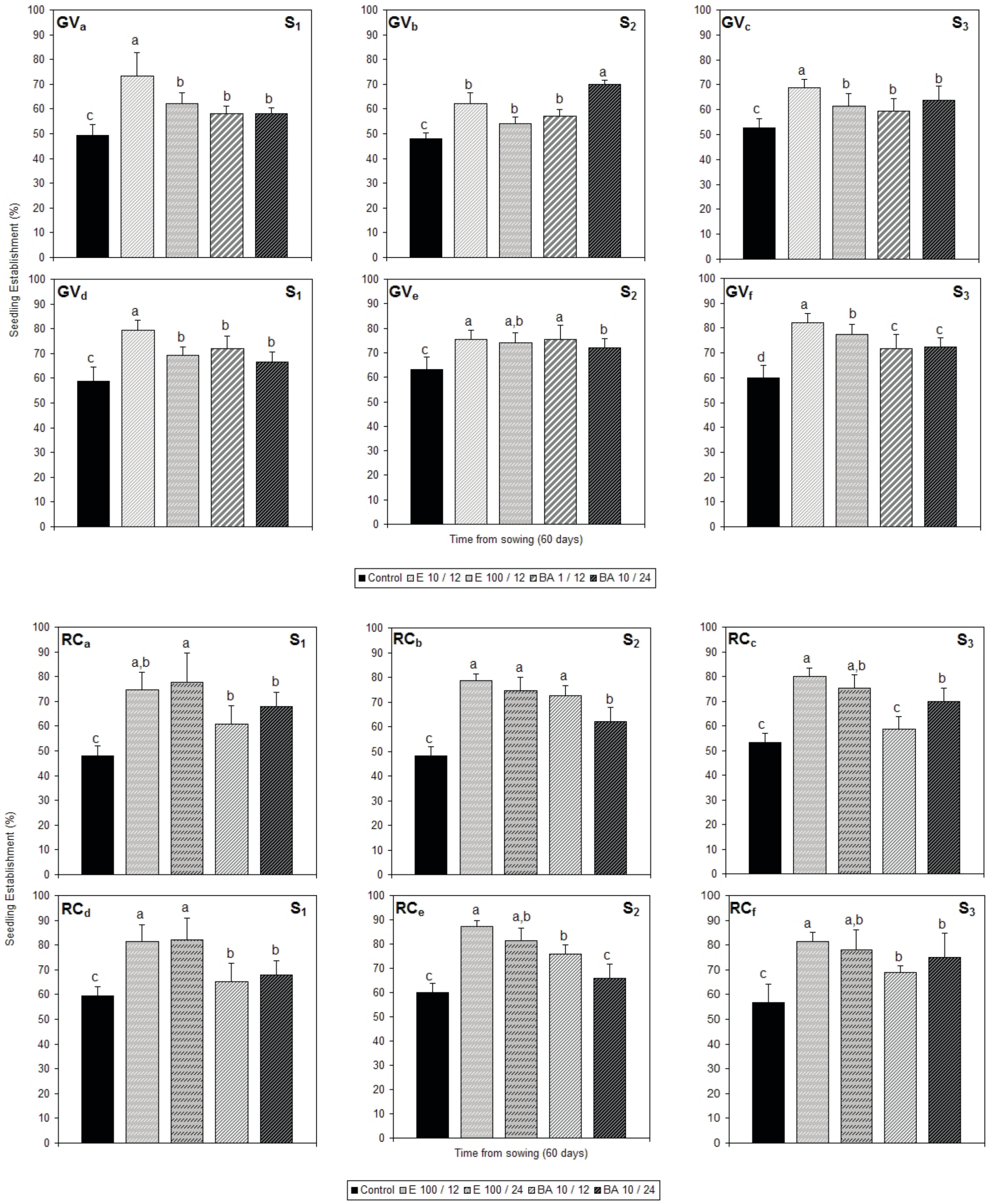

Figure 1. Seedling establishment percentage of *G. versicolor* (GV) and *R. complicata* (RC) under different plant growth regulators pre-treatments ($PGR_{ppm\ /\ immersed\ time\ (h)}$) in three soils ($S_1$, S_2, S_3) measured 60 days from sowing, in first (a, b, c) and second (d, e, f) experiments, respectively. Data shown are the mean from three replicates of 750 seeds each ± S.E. Different letters show means that are significantly different at $P<0.05$.

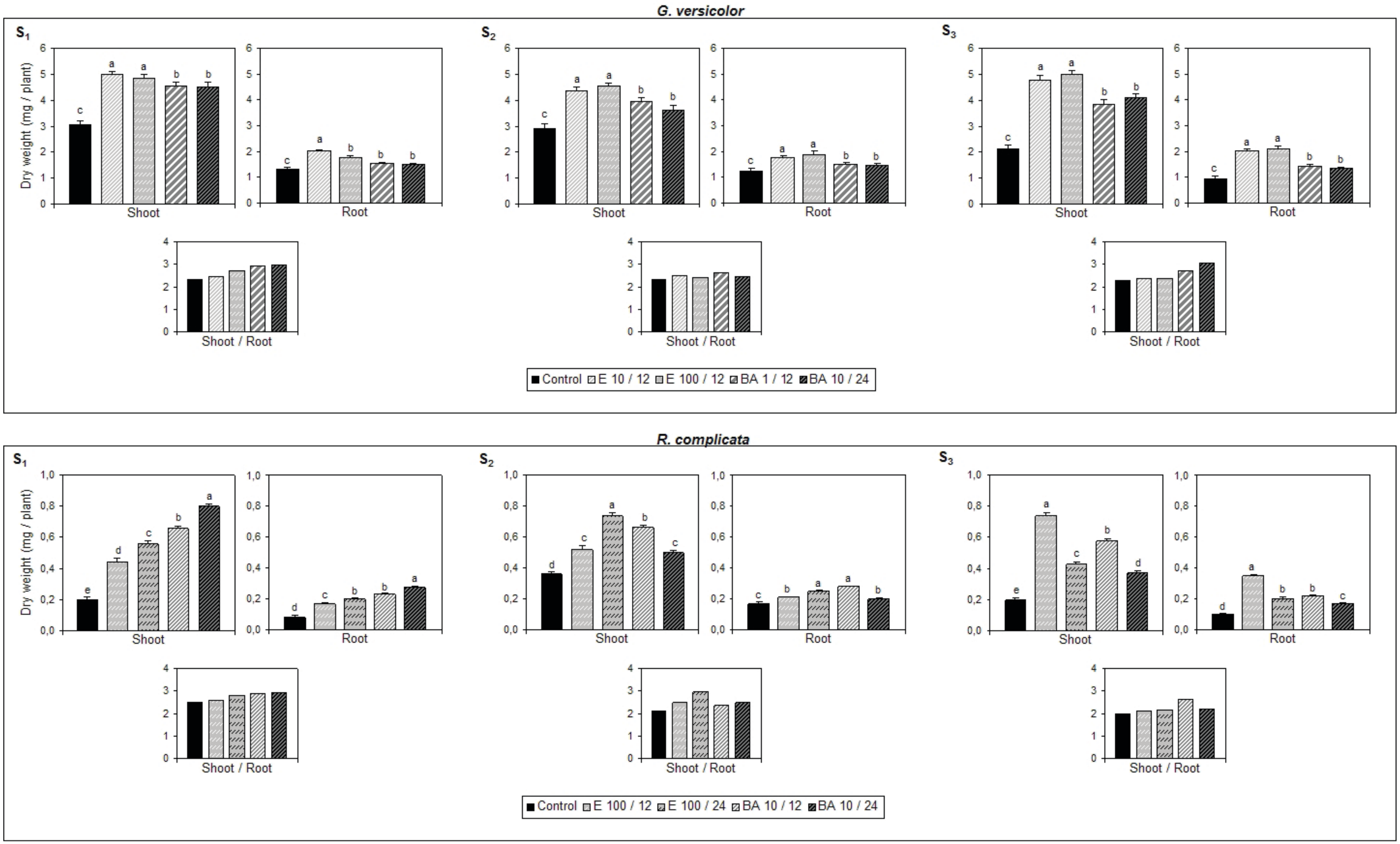

Figure 2. Dry weight and shoot/root relation of plants of *G. versicolor* and *R. complicata*, under different PGRs pre-treatments ($PGR_{ppm\,/\,immersed\ time\ (h)}$) measured after 60 days from sowing in three soils (S_1, S_2, S_3) mixed with quartz sand (4:1) and perlite. Data shown are the mean from three replicates of 750 seeds each ± S.E. Different letters show means that are significantly different at $P<0.05$.

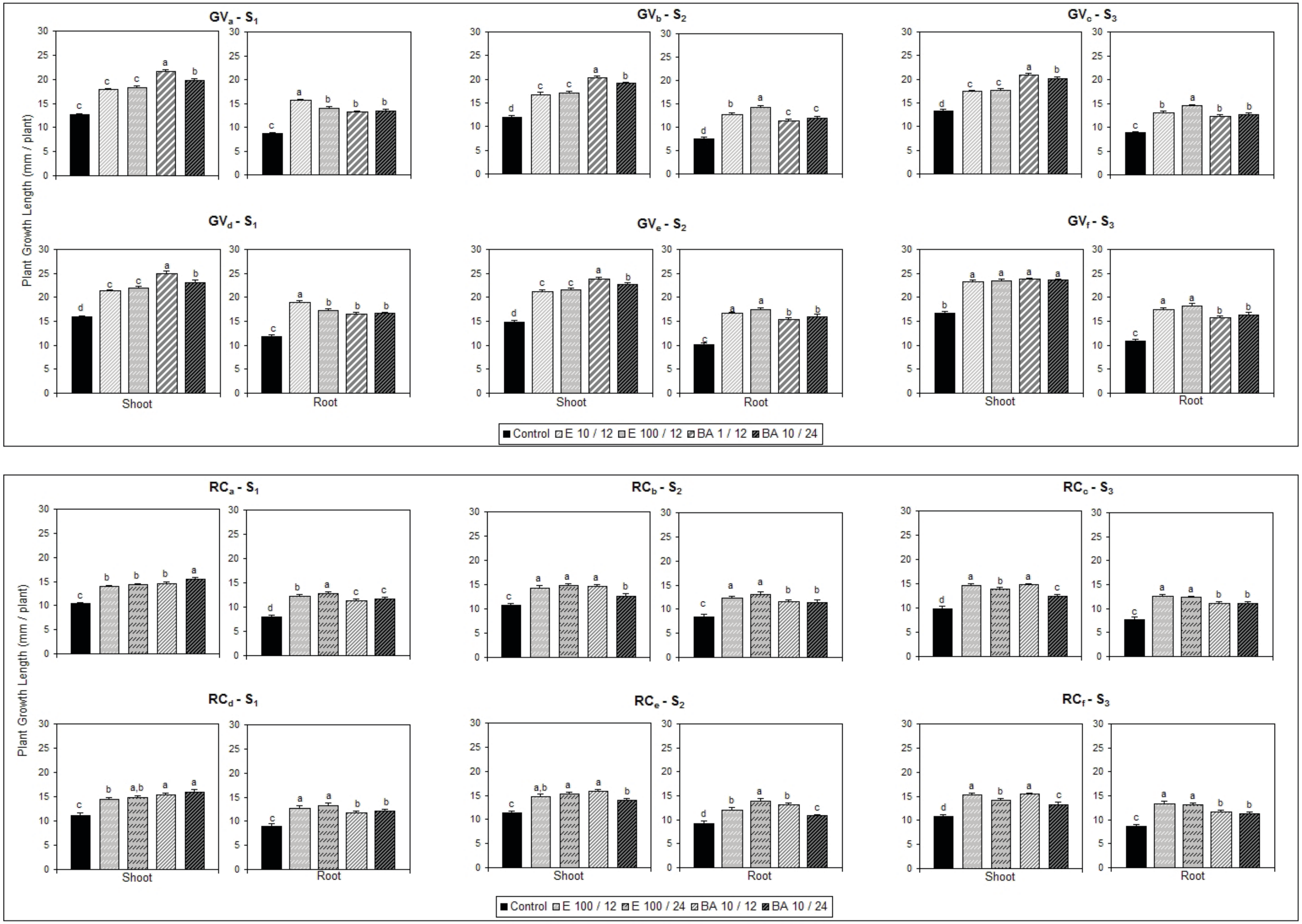

Figure 3. Plant Growth Length (mm) of plants of *G. versicolor* (GV) and *R. complicata* (RC) under different PGRs pre-treatments ($PGR_{ppm\,/\,immersed\ time\ (h)}$) in three soils ($S_1$, S_2, S_3) measured 60 days from sowing, in first (a, b, c) and second (d, e, f) experiments, respectively. Data shown are the mean from three replicates of 750 seeds each ± S.E. Different letters show means that are significantly different at $P<0.05$.

soil. This mixture significantly reduced soil compaction and therefore facilitated a greater movement of water and nutrients. From these results, it will be essential to till the soil before future revegetation to lower soil compaction and improve the seedling establishment and plant growth [29,30].

Furthermore, soil compaction can alter clevels ofplant growth regulators. A rise in soil compaction, leads to a significant increase in the levels of ABA and ethylene. In the case of ABA, the compaction rapidly induces an increase in the xylem sap [31], while ethylene increases its production at the basal level of the root, in response to mechanical barriers in the soil, which impede such growth [32].

The seedling establishment percentages were measured for each species. The possible water unavailability due to soil compaction, is offset by the rise in ethylene synthesis from the first few hours of imbibition. This may be the reason why low concentrations of exogenous Et are adequate to boost seedling establishment in less compact soils or those with greater nutrient availability [25,33].

Any unfavourable situation caused in plants, like soil compaction, raising the ABA levels, diminishes cytokinins. The experimental conditions may indicate that the BA concentrations used were adequate to reverse the inhibitory effects of ABA. The data confirms that the presence of ethylene promoted root elongation and stem growth [34-36]. Nevertheless, Pérez-López and colleagues suggest that the faster ethylene production by seedlings could provoke slower root and shoot elongation [37]. This appeared to occur in seedlings of both species and would account for the fact that with the Et application the root and shoot dry weight often exceeded that of the rest of the treatments, being, however, smaller in shoot size. This is due to the fact that Et, as it releases ethylene, promotes the reorientation of the microfibrils. Consequently, cellular growth is radial and thus the reduction in the length of the branches does not imply reduction in the biomass of the roots, stems and leaves [38]. Also, CO_2 and ethylene may interact in germination and growth [39]. This may occur by ethylene biosynthesis and axial root growth - breaking thermodormancy and subsequently raising the germination percentage [40]. This gaseous exchange, however, and its action on the seeds, depends on the sowing depth and thickness of the substrate cover, among other factors [39,41].

The growth of seedlings treated with BA were generally more effective in the second experiment as the result of greater soil aeration [42]. The drying of the roots lowers the endogenous Cks levels and increases after rehydration [43], when exogenous BA is applied in moderate concentrations [44]. If the action of the Cks was very positive on root formation and development, it is possible that the differences in stem elongation may be dependent on external parameters such as light [45]. Thus, it appears that light increases the presence of Cks in the stem and in the cotyledons. Hammerton and colleagues also propose that there is an association between the Cks content of the plants, their biomass, and presumably their photosynthetic potential when light is present [46]. These findings indicate that the stimulation of the expansion of the stem, cotyledons, and leaves could be the result of increased Ck levels in this study [47].

In the present study, the effectiveness of the administration of these two PGRs was verified in aspects as important for seedling development as soil properties, root formation, stem elongation, and leave growth. This raises the expectation that, when transferred to the field, these treatments will be an effective aid to plant recruitment in recovery programs.

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