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Application of hypergravity in *Eucalyptus* and *Corymbia* seeds

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ABSTRACT: *The aim of this study was to evaluate the growth responses of various Eucalyptus and Corymbia species subjected to different intensities of simulated hypergravity relative to the control. A centrifuge was used to simulate hypergravity. It was developed and built at the Centro de Microgravidade of the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil. Seeds of five Eucalyptus and one Corymbia species (E. grandis, Eucalyptus globulus, Eucalyptus benthamii, Eucalyptus saligna, Eucalyptus dunnii, and C. maculata) were placed on moist germination paper in plastic containers and rotated at speeds simulating 5 Gz and 7 Gz for different lengths of time. Hypergravity technology significantly increased seedling production (diameter, height, and survival at 120 days) in nurseries. In E. globulus, the effects of hypergravity were significant at 7 Gz at all lengths of time (from 1 d to 9 days). Effects of hypergravity were significant in both E. benthamii and E. grandis at 7 Gz and 8 h exposure. Therefore, simulated hypergravity could be used in performance tests of Eucalyptus seedlings in early stages of development.*
Key words: *rowth increment; forest breeding; simulated-gravity.*

Aplicação da hipergravidade em sementes de *Eucalyptus* e *Corymbia*

RESUMO: *O presente trabalho objetivou avaliar o crescimento de espécies de Eucalyptus e Corymbia em diferentes intensidades de hipergravidade simulada em relação ao controle. Uma centrífuga foi usada para simular a hipergravidade. Este equipamento foi desenvolvido e construído no Centro de Microgravidade da Pontifícia Universidade Católica do Rio Grande do Sul, Brasil. Sementes de cinco espécies de Eucalyptus e uma de Corymbia (E. grandis, E. globulus, E. benthamii, E. saligna, E. dunnii, e C. maculata) foram colocadas em papeis de germinação e em recipientes plásticos, em que foram rotacionadas a velocidades simuladas de 5 Gz e 7 Gz, por diferentes períodos de tempo. A tecnologia da hipergravidade proporcionou aumento significativo na taxa de crescimento das plântulas (diâmetro, altura e sobrevivência aos 120 dias) no viveiro. Para Eucalyptus globulus, os efeitos da hipergravidade foram significativos na intensidade de 7 Gz em qualquer período de tempo (do primeiro até o nono dia). Os efeitos da hipergravidade foram significativos para as espécies E. benthamii e E. grandis na intensidade 7 Gz e 8 horas de exposição. Dessa maneira, a hipergravidade simulada apresenta potencial de uso em testes com plântulas de eucaliptos em estágios iniciais de desenvolvimento.*
Palavras-chave: *incremento de crescimento; melhoramento do Eucalyptus e Corymbia; gravidade simulada.*

INTRODUCTION

Eucalyptus improvement has placed the paper and cellulose divisions of the Brazilian forestry industry in a prominent position. Advances in productivity and wood quality are largely due to the development of interspecific *Eucalyptus* hybrids and the cloning of the best individuals from progeny (ASSIS & MAFIA, 2007). Hybrids between *Eucalyptus urophylla* x *E. grandis* have been produced with favorable adaptation traits, rooting ability, resistance to *Eucalyptus* canker disease, and high yield (ASSIS et al., 1993; ASSIS & MAFIA, 2007; BATISTA et al., 2014; CASTRO et al., 2016).

RESENDE & ASSIS (2008) proposed reciprocal recurrent selection between synthetic multi-species populations (RRS-SMSP). In this method, genetically superior individuals are obtained with

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desirable traits present in several species. In the RRS-SMSP technique, two synthetic multi-species populations are obtained and recurrent selection is performed between them. In this way, it is possible to congregate desirable traits belonging to several species in an individual. Heterosis is exploited for growth by using several pairs of species whose combinations are known to be heterotic (RESENDE & ASSIS, 2008).

RRS-SMSP is widely used in *Eucalyptus* improvement programs and this method has proven to be effective (ASSIS & MAFIA, 2007; RESENDE & ASSIS, 2008; CASTRO et al., 2016). Nevertheless, much time is required to conduct controlled breeding, obtain seeds, and assemble hybrid progeny tests. Therefore, methods are needed to obtain superior individuals in the shortest time possible and maximize genetic gains from the selection process. It has been reported that simulated hypergravity rapidly increases plant growth and the number of germinated seeds. It requires only that plant propagules be subjected to higher gravitational acceleration forces (G-force) than those on earth (RUSSOMANO et al., 2007; SANTOS et al., 2012).

Hypergravity can be simulated by using a centrifuge that combines several forces and produces a G-force that acts on the specimen. During centrifuge rotations, normal force balances the weight force while the centripetal force pulls the moving body towards the center of the circle. Inertia keeps it in the tangential direction. The resulting force is related to the G effect applied to the body (RUSSOMANO et al., 2007). It occurs in magnitudes higher than the Earth's gravitational force (1Gz) and may cause phenotypic changes in the plants subjected to it. It may also change seed germination patterns (HOSON et al., 1996; SOGA et al., 1999; NAKABAYASHI et al., 2006; RUSSOMANO et al., 2007; MATSUMOTO et al., 2010; MANZANO et al., 2012; SANTOS et al., 2012; HOSON, 2014).

Effects of simulated hypergravity on radish and cucumber (KASAHARA et al., 1995), watercress (HOSON et al., 1996), beans (SOGA et al., 1999), carrot (SANTOS et al., 2012), rocket (RUSSOMANO et al., 2007), *Arabidopsis thaliana* (NAKABAYASHI et al., 2006; MANZANO et al., 2012), and other *Arabidopsis* species (MATSUMOTO et al., 2010) have been studied. However, as yet, to our knowledge, no studies have reported the use of hypergravity to increase the growth and germination of *Eucalyptus*.

The aim of this study was to evaluate the growth responses of various *Eucalyptus* species subjected to different intensities of simulated hypergravity relative to the control (1Gz) in early stage of development (seedlings).

MATERIALS AND METHODS

The simulated hypergravity experiment was conducted at the Laboratório de Engenharia Aeroespacial Joan Vernikos and Centro de Microgravidade at the Pontifícia Universidade Católica do Rio Grande do Sul (FENG/PUCRS). Seed planting, management and assessment were carried out in 2015 at the nursery of the CMPC Celulose Riograndense Company, located in the municipality of Barra do Ribeiro, RS, in the Barba Negra Forestry Park (latitude 30°17′S, longitude 51°18′W and altitude 12m). According to the Köppen classification, the prevailing climate in the region is of the Cfa type (humid subtropical) with an average annual rainfall of about 1400mm. The average temperature of the warmest month does not exceed 25°C and the coldest month 14°C, with light frosts.

Plant material

A centrifuge designed and developed at the Centro de Microgravidade, PUCRS, was used to simulate hypergravity. Several *Eucalyptus* and one *Corymbia* species were used in this experiment. These included *E. grandis* (lot – 00049 PSM. HF.Dura RS/ex.qld – Australia), *E. globulus* (lot 00012 – Otwai Range – Australia), *E. benthamii* (PSM – Ipiranga, RS), *E. saligna* (lot 00028 - PSM – HF Jerônimo, RS/ex, QLD – NSW – Australia), *C. maculata* (lot 00002 - PSM – HF – Camélia, RS), and *E. dunnii* (lot 01 - PSM – CMPC). The CMPC Celulose Riograndense Company provided the plant materials and the experiments were conducted in 2015.

Exposure of seeds to intermittent simulated hypergravity – scenario 1

E. grandis and *E. globulus* seeds were placed on rectangular filter paper (18cm long by 7cm wide) moistened with water. Fifteen seeds were placed 1cm from the top edge of each filter paper. These were rolled and transferred into 300mL containers. Three filter paper rolls were placed in each container (45 seeds per sample). To each container, 40mL water was added. Each container was then covered with perforated plastic film to minimize evaporative water loss.

In this study, a simulated hypergravity of 7Gz was applied intermittently for 1, 2, 3, 4, 5, 6, 7 and 9d. The cycle was 8h in simulated hypergravity and 16h at rest (1Gz). Each protocol had a control group at rest (1Gz) under the same light and humidity conditions throughout the experiment. Protocols were carried out in triplicate.

After treatment with a simulated hypergravity of 7Gz at different exposure periods, the seeds of *E. grandis* and *E. globulus* were taken to a greenhouse to evaluate their survival rates (number of plants surviving out of 45 sown seeds), plant height (cm), plant diameter (cm), number of roots (nº roots), root wet weight (WW, g), and root dry weight (DW, g). Seeds were planted in a completely randomized block design. Seeds were planted in polypropylene tubes with a volume of 55cm^3 and the substrate was composed of 20% vermiculite and 80% Canadian peat. The base fertilizer of the substrate was a mixture containing PG mix™, Super simple (phosphate) and Osmocote® (Scotts Miracle-GroCompany) (NPK 19:06:10), in a dosage concentration of 2.0kg, 2.0kg and 1.5kg m^{-3} of substrate, respectively. The greenhouse was equipped with a misting irrigation system using sprays nozzles with a flow rate of 7.5L h^{-1}, with automated control of moisture and water table irrigation. In all stages, the irrigation intensity varied according to weather conditions. For survival evaluation, 45 seeds were sown in triplicate in the greenhouse. Surviving plants were counted in the first- and second months after sowing. Four-month-old seedlings were sampled and evaluated in triplicate for growth traits, number of roots, WW, and DW. Four representative individuals were then selected from each replicate for evaluation.

Exposure of seeds to uninterrupted simulated hypergravity – scenario 2

E. grandis, *E. globulus*, *E. benthamii*, *E. saligna*, *C. maculata*, and *E. dunnii* seeds were placed on filter paper and into plastic containers as described previously. They were subjected to continuous 7Gz and 5Gz for 8h and 24h. The control group consisted of seeds that did not receive hypergravity treatment. This procedure was repeated three times for each combination of G effect and exposure time. Sizes of the shoots and roots were recorded in the laboratory on day ten for all species.

After the 5Gz and 7Gz hypergravity treatments for 8h and 24h, seeds were taken to the greenhouse to rate survival, plant height, plant diameter, number of roots, root wet weight, and root dry weight. Seeds were also planted in a completely randomized block design at the same nursery conditions described above. For the survival evaluation, 45 seeds were sown in triplicate in the greenhouse. Surviving plants were counted during the first- and second months after sowing. To evaluate the four-month-old plants, four representative individuals from each of the three replicates were chosen according to their growth traits, number of roots, WW, and DW.

Statistical analysis

The data were tested for normality using the Shapiro-Wilk test (1965), and each treatment was analyzed. The data were normally distributed at 5% probability level. To evaluate the laboratory and nursery data, a completely randomized block design in a factorial arrangement was used. The main factors were hypergravity intensity and exposure time. The interaction was hypergravity intensity x exposure time, and the treatments were the various combinations of hypergravity intensity and time. An analysis of variance (ANOVA) was performed on each variable. The significance of each factor was assessed separately using Duncan's test at a 5% probability level. The significance of the interaction between gravity intensities and different timespans was also assessed. Scenarios 1 and 2 were considered separately in the statistical analysis. Statistical analyses were carried out with R Software (R Core Team, 2016).

RESULTS AND DISCUSSION

Exposure of seeds to intermittent simulated hypergravity – scenario 1

7Gz hypergravity applied intermittently to *E. globulus* seeds increased nursery seedling survival, height, and diameter relative to the control (Table 1). In the 7Gz treatment of *E. globulus* seeds, seedlings survival, height, and diameter were about 55%, 12%, and 14% greater than those of the untreated (control) seeds, respectively.

RUSSOMANO et al. (2007) tested the effect of intermittent exposure of *Eruca sativa* Mill (rocket) to 7Gz. They reported that the hypergravity-treated seeds had higher germination potential than the untreated controls. Whereas untreated rocket seeds germinate within 4-7d, seeds treated with 7Gz germinated in only 3d. The authors also noted that seeds exposed to intermittent 7Gz produced seedlings that grew taller (average 3.2cm) than those derived from untreated control seeds (average 1.9cm). These data corroborated the results of this study (*E. globulus* seeds treated with 7Gz produced plants with greater diameters, heights and survival times than the controls, Table 1).

According to RUSSOMANO et al. (2007), hypergravity modifies auxin translocation in plants and increases their height relative to untreated controls. Auxin elongates subapical cells and affects phototropism, geotropism, and fruit development. Simulated hypergravity may have increased cell wall acidification, an important cell

Table 1 - Means and statistical significance of intermittent *E. grandis* and *E. globulus* exposures to 7 G for 1, 2, 3, 4, 5, 6, 7, and 9 d (scenario 1) for survival (number of surviving plants derived from 45 seeds), plant height (cm), plant diameter (cm), number of roots (nº roots), root wet weight (WW, g), and root dry weight (DW, g).

Variable	----------------------*E. grandis*---------------------			--------------------*E. globules*-----------------		
	1Gz*	7Gz	p-value	1Gz*	7Gz	p-value
Survival (number of plants)	21.66	24.66	89% NS	6.58	10.21	0.0053%S
Height (cm)	22.62	23.13	33% NS	12.53	14.07	0.18%S
Diameter (cm)	2.55	2.82	24% NS	2.33	2.65	0.018%S
Roots (number)	6.41	6.96	42% NS	5.22	6.54	7%NS
WW (g)	2.41	2.17	26% NS	3.32	3.33	98%NS
DW (g)	0.43	0.42	96% NS	0.53	0.64	12%NS

*Control; S: effect of 7 G significant at 5% probability level by the F-test; NS: effect of 7 G not significant at 5% probability level by the F-test; The time factor and the interaction between time and G intensity were not significant at the 5% probability level.

growth process that enhances the activity of the enzyme associated with cell wall malleability and elongation. Osmotic pressure forces water into the cell and makes it expand (RUSSOMANO et al., 2007).

There were no significant differences among the various hypergravity exposure time. This result demonstrated that for *E. globulus* species, the application of 7 Gz force worked from 24h intermittent exposure (8h exposure to 7Gz and 16h at rest) for the variables of survival, height and seedling diameter. Interaction between exposure time and hypergravity intensity was not significant. Therefore, exposure time and hypergravity intensity act independently in *Eucalyptus*.

Exposure of seeds to uninterrupted simulated hypergravity – scenario 2

There was no statistical significance in relation to the increase of plant growth due to hypergravity for any of the *Eucalyptus* species or variables evaluated in the laboratory (Table 2). Duncan's test was carried out to compare the means of the centrifugal groups (seeds that received 7Gz treatment) and control groups (1Gz). There were no increases in aerial shoot height (AP, cm) or root length (root S, cm) due to the application of 7Gz. Nevertheless, both shoot- and root growth were relatively higher in the untreated control (Table 2). *E. saligna* roots were 43% higher in the control than the centrifugal group. For *C. maculata*, 43%

Table 2 - Means of the main factors studied for 8 h and 24 h continuous 7 Gz and 5 Gz treatments (scenario 2) and the respective control groups for *E. grandis*, *E. globulus*, *E. benthamii*, *E. saligna*, *C. maculata*, and *E. dunnii*. The variables evaluated in laboratory were aerial shoot height (AP, cm) and root length (root S, cm).

Hypergravity intensities and controls	-------------*E. saligna*----------		----------*C. maculata*--------		-----------*E. benthamii*----------	
	AP (cm)	Root S (cm)	AP (cm)	Root S (cm)	AP (cm)	Root S (cm)
7 Gz	0.44a	0.46b	0.93b	1.15c	1.01a	1.17a
Control	0.56a	0.66a	1.15b	1.65b	1.04a	1.22a
5 Gz	0.48a	0.39b	1.64a	1.92b	0.91b	1.21a
Control	0.47a	0.45b	1.82a	2.35a	0.88b	1.26a
Hypergravity intensities and controls	-------------*E. dunnii*----------		-----------*E. globulus*--------		------------*E. grandis*-----------	
	AP (cm)	Root S (cm)	AP (cm)	Root S (cm)	AP (cm)	Root S (cm)
7 G	1.34a	1.28a	0.81ab	1.02ab	0.74a	0.78a
Control	1.25a	1.40a	0.94a	1.37a	0.84a	0.85a
5 G	1.31a	1.19a	0.80ab	1.10ab	0.77a	0.64b
Control	1.20a	1.33a	0.58b	0.76b	0.79a	0.77ab

Means for each species and variable followed by the same vertical letters do not significantly differ according to Duncan's test at a 5% probability level. The main effect of time and the interaction between time and Gz intensity were not significant according to the ANOVA F-test.

and 22% increases were measured for Root S in both control groups, respectively. These results showed that we can predict neither the direction nor the magnitude of the seedling responses to simulated hypergravity.

Application of 7Gz to *E. benthamii* and *E. grandis* seeds had a statistically significant effect on the seedlings derived from them in the nursery (Table 3). *E. benthamii* seeds exposed to 7Gz produced seedlings with significantly greater

Table 3 - Means of the main factors studied for 8 h and 24 h continuous 7 Gz and 5 Gz treatments (scenario 2) and the respective control groups for *E. grandis*, *E. globulus*, *E. benthamii*, *E. saligna*, *C. maculata* and *E. dunnii.* Variables evaluated in the nursery were: survival (number of surviving plants derived from 45 seeds), plant height (cm), plant diameter (cm), number of roots (nº roots), root wet weight (WW, g), and root dry weight (DW, g).

Hypergravity intensities and controls						
	------*E. saligna*------					
	Survival (number)	Height (cm)	Diameter (cm)	Roots (number)	WW (cm)	DW (cm)
7 G	0.83^{b}	18.05^{b}	2.52^{a}	6.66^{b}	2.08^{b}	0.36^{a}
Control	0.66^{b}	16.95^{b}	2.33^{a}	6.33^{b}	1.64^{b}	0.40^{a}
5 G	3.16^{b}	20.5^{a}	2.56^{b}	8.66^{a}	2.80^{a}	0.37^{a}
Control	6.5^{a}	21.5^{a}	2.38^{b}	10.08^{a}	2.81^{a}	0.34^{a}
	------*C. maculata*------					
	Survival (number)	Height (cm)	Diameter (cm)	Roots (number)	WW (cm)	DW (cm)
7 G	10.83^{b}	20.37^{a}	2.77^{a}	5.0^{a}	3.34^{a}	0.64^{a}
Control	10.83^{b}	21.04^{a}	2.69^{a}	3.0^{ab}	1.71^{a}	0.35^{a}
5 G	17.16^{a}	18^{b}	2.70^{a}	2.0^{ab}	2.00^{a}	0.42^{a}
Control	16.33^{a}	17^{b}	2.60^{a}	2.67^{b}	2.27^{a}	0.53^{a}
	------*E. benthamii*------					
	Survival (number)	Height (cm)	Diameter** (cm)	Roots (number)	WW (cm)	DW (cm)
7 G	13.83^{a}	20.91^{a}	2.63^{a}	5.16^{b}	2.43^{a}	0.41^{a}
Control	11.66^{a}	19.29^{a}	2.28^{b}	4.33^{b}	1.92^{b}	0.33^{a}
5 G	8.33^{b}	20.91^{a}	2.36^{b}	6.5^{a}	2.38^{a}	0.37^{a}
Control	2.33^{b}	19.29^{a}	2.42^{b}	7.5^{a}	2.57^{a}	0.28^{a}
	------*E. dunnii*------					
	Survival (number)	Height (cm)	Diameter (cm)	Roots (number)	WW (cm)	DW (cm)
7 G	7^{b}	17.25^{a}	2.46^{a}	9.83^{a}	2.77^{a}	0.37^{a}
Control	5^{b}	15.62^{a}	2.38^{a}	9.00^{a}	2.90^{a}	0.43^{a}
5 G	22.33^{a}	17.87^{a}	2.45^{a}	6.66^{a}	2.17^{a}	0.29^{a}
Control	19.33^{a}	18.91^{a}	2.43^{a}	7.58^{a}	3.00^{a}	0.35^{a}
	------*E. globulus*------					
	Survival (number)	Height (cm)	Diameter (cm)	Roots (number)	WW (cm)	DW (cm)
7 G	1.16^{b}	-	-	-	-	-
Control	0.16^{b}	-	-	-	-	-
5 G	9.5^{a}	-	-	-	-	-
Control	6.66^{a}	-	-	-	-	-
	------*E. grandis*------					
	Survival* (number)	Height (cm)	Diameter (cm)	Roots (number)	WW (cm)	DW (cm)
7 G	16.66^{a}	24.20^{a}	2.64^{a}	3.16^{b}	3.46^{a}	0.53^{a}
5 G	10.16^{c}	19.33^{b}	2.42^{b}	8.16^{a}	2.09^{a}	0.31^{a}
Control	11.83^{c}	24.33^{a}	2.38^{b}	8.33^{a}	2.28^{a}	0.35^{a}

Means followed by the same vertical letters do not significantly differ by Duncan's test at a 5% probability level. – death of the plant; *Significant effects of 7 G (individual) and time (individual) (P = 0.001%). Plant survival rates were highest when seeds exposed to hypergravity for 24 h. No interaction was reported between gravity intensity and exposure time; **Significant effect of 7 G (individual). Effect of time (individual) not significant (*p* = 70%). No interaction wasreported between gravity intensity and exposure time.

seedling diameters than those of the controls according to Duncan's test at the 5% probability level (Table 3). Seedlings treated with 7Gz were 15% larger in diameter than the untreated controls.

Seed treatment with 7Gz uninterrupted for 8h and 24h increased *E. grandis* seedling survival relative to controls according to Duncan's test at a 5% probability level (Table 3). For *E. grandis,* the 7Gz treatment increased seedling survival by 28% more than the control (Table 3). In addition, the time effect was significant by the ANOVA F-test at a 5% probability level: the survival rate was highest with the 8h exposure time (Table 3).

SANTOS et al. (2012) evaluated the germination potential of *Daucus carota* L. exposed to 7Gz for 8h followed by 16h rest. This procedure was repeated for four consecutive days. The authors reported that a higher percentage of carrot seeds treated with 7Gz germinated than the untreated controls. The 8.89%, 12.35%, and 15.06% increases in germination rate relative to the controls were obtained for hypergravity-treated carrot seeds on the fourth, eleventh, and eighteenth days of evaluation, respectively. These results corroborated the findings of this present study.

Very high magnitudes of hypergravity affected plant propagule growth. HOSON et al. (1996) reported that growth suppression occurred in watercress (*Lepidium sativum* L.) seedlings derived from seeds exposed to gravity exceeding 35Gz. According to these authors, hypergravity at high levels causes cell walls to thicken and stiffen, and increase the lignin content. These alterations in cell wall metabolism inhibit cell growth and elongation.

KASAHARA et al. (1995) studied the effects of hypergravity on radish and cucumber and concluded that plant growth was reduced at 20Gz. Nevertheless, the growth of seedlings subjected to 13Gz was not affected, and did not significantly differ from that of the control. MATSUMOTO et al. (2010) studied the effect of hypergravity on *Arabidopsis thaliana* and concluded that 300Gz suppressed growth. Gravitational conditions affect the morphology and mechanical properties of xylem vessels (NAKABAYASHI et al., 2006). As hypergravity intensifies, polysaccharides deposition increases, cells become more rigid and their growth is inhibited (SOGA et al., 1999). Therefore, the hypergravity intensities applied in the present study suffice to increase *Eucalyptus* growth and yield.

Statistically significant increases in *E. benthamii* diameter in response to 7Gz exposures may mean enhanced short-term growth and a breakthrough in *Eucalyptus* improvement. MANZANO et al. (2012) obtained higher *Arabidopsis thaliana* seedling lengths after subjecting seeds to 2Gz and 6Gz for 4d. They attributed the increase in length to changes in cell wall components. High levels of gravity may modify physiological processes by inducing mutations and/ or epigenetic changes (i.e., phenotypic alteration that does not result from changes in the DNA nucleotide sequence; ALBERTS et al., 2015).

Modifications caused by genetic and/ or epigenetic factors may benefit, injure or kill plants derived from seeds exposed to hypergravity. According to the present study, the mechanism by which these changes occur varies with plant species. Unlike the other *Eucalyptus* species, 7Gz hypergravity increased *E. grandis* survival rates relative to untreated controls. As discussed in scenario 1, then, it is not yet possible to predict the direction or the magnitude of the effect caused by the application of hypergravity in *Eucalyptus*.

Unlike the laboratory data, those obtained from the nursery experiments were statistically significant. Therefore, it is assumed that the effect of simulated hypergravity is expressed throughout the *Eucalyptus* growth cycle. It is expected that even stronger effects of hypergravity would be observed in field trials using trees old enough to be harvested.

CONCLUSION

Simulated hypergravity technology enabled significant increases in nursery *Eucalyptus* seedling production (in terms of diameter, height, and survival at 120 d) for different species and at various hypergravity intensities and durations. Based on the results obtained in the present study, it is feasible that simulated hypergravity could be used to improve *Eucalyptus* growth and merits further investigation.

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