

The Effect of Slow-grown in Vitro Storage Under Different Light Spectra on Banana Plantlets Cv. Prata Catarina (AAB).

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1 **The effect of slow-grown *in vitro* storage under different light spectra on banana plantlets cv. Prata Catarina (AAB).**

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14 **Keywords:** tissue culture, acclimatization, somaclonal variation, plantlet, LED

15
16 **Abstract**

17 Maintaining updated *in vitro* plant subcultures is essential for commercial micropropagation and tissue
18 culture research. In unusual situations, the subcultures can be delay and the slow-growth *in vitro* storage technic could
19 be applied to reduce the loss of plant material. The present study aimed to evaluate the slow-growth *in vitro* storage
20 of banana plantlets ('Prata Catarina'; group AAB) under different light spectra. Shoot cultures in MS medium without
21 plant growth regulators were maintained under blue (B), red (R), red plus blue (R2B), and white (CW) light spectra
22 ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$; $50 \mu\text{mol m}^{-2} \text{s}^{-1}$) for up to 140 days. The plantlets maintained under the R, CW, and R2B spectra did not
23 survive after 140 days of *in vitro* slow-growth storage. The plantlets maintained under the B spectrum survived after
24 140 days of *in vitro* slow-growth storage and showed little browning.

25
26 **Abbreviations:** MS - Murashige and Skoog medium, BA - 6-Benzylaminopurine, CML – Commercial
27 micropropagation laboratory, SGS – Slow grown storage, LS – Light spectra.

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29
30 In commercial micropropagation laboratories (CML), operational difficulties may arise during commercial
31 micropropagation processes when the *in vitro*-produced plantlets that are ready to be transplanted cannot be moved to
32 the greenhouse because they are full of unsold plants or because the customer is not ready to receive the plants. Such
33 operational difficulties can result in financial losses to both CMLs and customers (Watt et al. 2000). Recently, many
34 CMLs and tissue culture research centers (TCRC) were temporarily closed due to the COVID-19 pandemic, which
35 reduced the workforce, thereby limiting the subculture frequency during the lockdown. In such unusual situations,
36 CMLs and TCRCs can employ *in vitro* slow-growth storage (SGS).

37 During *in vitro* SGS, to increase the period before subcultures, the development of plants is retarded by
38 suppressing their metabolism, albeit without altering the genetic uniformity and quality of micropropagated plantlets
39 (Kamińska et al. 2016). Culture conditions (temperature, photoperiod, and light intensity) and culture medium
40 components (organic and inorganic nutrients, osmotic, and plant growth regulators) can be adjusted to suppress plant
41 metabolism (Oliveira et al. 2000; Gopal and Chauhan 2010; Kaur et al. 2012; De Carvalho et al. 2014; Thakur et al.
42 2015).

43 In the banana germplasm collection at the International Transit Centre (ITC) in Belgium, over 1,600 banana
44 accessions sourced from 38 countries have been maintained under *in vitro* SGS. At ITC, *in vitro* SGS is achieved
45 under a combination of low temperature (16°C) and limited light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) in a growth room (Van den houwe
46 et al. 1995; Panis et al. 2020). Therefore, this method is appropriate to decrease the frequency of subculture by
47 retarding the growth of plantlets, ranging between 3 and 22 months, depending on the genomic group and *in vitro*
48 browning reaction (Panis et al. 2020). At banana CMLs the limiting of subculture frequency is shorter (around 3-4
49 weeks). Between 12 and 16 weeks *in vitro* does not update the subculturing cause severe explant damages. The
50 cytokinin stops working, and the shoots start rooting until consuming all the culture media. In this case, the laborers
51 spend triple times working to cut the roots, and the frequency of contamination increase (Pers. Comm).

52 To date, few studies have explored different light spectra for *in vitro* SGS. The use of different light spectra
53 for *in vitro* SGS of *Heliconia champneiana* 'Splash' plantlets was evaluated and promising outcomes were achieved
54 using the blue spectrum for up to 12 weeks (Rodrigues et al. 2018). To the best of our knowledge, however, there are
55 no reports on the use of this technique for banana tissue culture.

To this end, the present study proposes to evaluate the effect for banana *in vitro* SGS under the different light spectra without compromising plantlet quality.

Fifty shoot tips (ST) were obtained from the uniform sword suckers of 'Prata Catarina' (SCS451; group AAB) with 3–4-month age. The STs were introduced in test tubes (one ST per tube) containing a semi-solid culture medium MS salts and vitamins (Murashige, T.; Skoog, F. 1962) plus 30 g L⁻¹ sucrose, 1.0 mg L⁻¹ 6-benzylaminopurine (BA; Sigma-Aldrich), and 2.0 g L⁻¹ Phytigel® (Sigma-Aldrich); the pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 121°C. The STs were incubated in a growth room for 30 days at 25°C ± 2°C, photoperiod of 14 h; white light was supplied from an LED (6000k, Philips®) at a photosynthetic photon flux density (PPFD) of 50 μmol m⁻²s⁻¹ (LI-250A, LI-COR). Forty healthy STs were induced to multiply under the same conditions as described above, but in jars containing 40 ml of a semi-solid culture medium MS salts and vitamins, plus 30 g L⁻¹ sucrose, 3.5 mg L⁻¹ BA, 2.0 g L⁻¹ Phytigel®, and subculture was performed four times. Few subcultures every 4 weeks ensured the genetic uniformity of the explants (Rodrigues et al, 1998).

Next, 400 *in vitro*-produced shoots were selected, cut to obtain uniform explants (1.5 cm × 0.5 cm), and individually inoculated into test tubes (15.0 cm × 2.5 cm) with a cap (2.5 cm; Kimble®) containing 7.0 mL of the semi-solid MS medium as described above but without plant growth regulators. The tubes were incubated under the same conditions as described above but distributed into four light treatments (GreenPower TLED®; Philips®): R = 100% red light (645-675 nm); B = 100% blue light (450-465 nm); R2B = 70% red + 30% blue light; and CW = white control (6000k, Philips®); the PPFD for all treatments was set at 50 μmol m⁻²s⁻¹. In each treatment, the test tubes were centered and equidistantly arranged to ensure uniform light incidence.

Each light treatment included 100 explants, divided into five-time intervals with 20 explants per interval (I1, I2, I3, I4, and I5) without subculture. Every 28 d, 20 plantlets from each treatment were evaluated for survival, height (from the base of the pseudostem to the cigar leaf tip, cm), fresh weight (g), pseudostem diameter immediately above the original cut (cm), number of leaves, number of primary roots, the longest root length (cm), and chlorophyll content (expressed as the SPAD index) of the third leaf from the top downward, measured using the CFL1030- chlorophyll meter (Falker®).

In vitro-stored plantlets evaluated at the indicated time points under each treatment were acclimatized in separate 24-cell plastic trays (163 cm³) containing a commercial horticulture substrate- BASE® (50% pine bark, 30% fibrous peat, and 20% vermiculite). The trays were arranged on 0.90 m high benches and centered in a greenhouse with fogging under 80%-90% humidity and 50% shading (Aluminet®). The temperature was maintained at 28°C ± 2°C during the 28 days acclimatization period. During this period, the plantlets were evaluated for monitored survival, plantlet height (cm), pseudostem diameter (cm), and leaf number.

During *in vitro* SGS, and acclimatization period, the experimental design was completely randomized, with plots representing the light treatments and subplots representing the time intervals. All data were subjected to analysis (*F* test) of variance in the split-plot arrangement, and the mean values among treatments and across time intervals were compared with Tukey's test at 5% significance.

During SGS, there was an interaction between plant height and light spectrum ($p = 0.0359$) (Fig. 1a). The cigar leaf length of explants increased steadily up to I4 but decreased during I5 (Fig. 2c). The height of explants grown under different light spectra was in the order B<R2B<CW<R (from the shortest to tallest) across time intervals (Fig. 1a).

During SGS, the different light spectra affected leaf number ($p = 0.0005$) and pseudostem thickness ($p = 0.0285$). These characteristics steadily increased until I5 (Fig. 1b and 1c). No symptoms of leaf senescence were observed under any light spectrum at I5 (Fig. 2a). The different light spectra affected fresh weight ($p < 0.0001$). During the experimental period, fresh weight was the lowest under the B spectrum, and the value fluctuated under the R spectrum during I4. In contrast, fresh weight was the highest under the CW and R2B spectra from I2 to I5 (Fig. 1d).

During SGS, there was an interaction between root number and light spectrum ($p < 0.0001$). During I2, there was a significant increase in growth under the R2B spectrum compared with that under the remaining spectra (Fig. 1d). The number of primary roots increased from I2 to I4 under all evaluated spectra, except R2B.

There were significant differences in evaluated root number under the CW and other spectra during I3 and I4. During I5, primary root number under the CW spectrum and growth under the R2B and R spectra were significantly reduced, but the values were stable under the B spectrum. Of note, under the CW spectrum, primary root number increased from I2 to I4 but decreased during I5.

There was a significant interaction between the length of the longest root (Fig. 1e) and the light spectrum ($p < 0.0001$). This length increased from I1 to I4 under all spectra, except CW. Under the CW spectrum, there was a significant reduction in this length from I2 to I3 and a further significant reduction from I3 to I4, and the values were lower than those under all the remaining spectra. During I5, there were significant reductions in the length of the longest root under the R, B, and R2B spectra, but the values were stable under the CW spectrum. The roots suffered

112 the effect of *in vitro* senescence over time, in the order of CW>R2B>R>B. Under the B spectrum, characteristic young
 113 root color was observed even after 140 days of *in vitro* SGS (Fig. 2a and red rectangle in 2b). Although we did not
 114 quantify the consumption of the culture medium, a larger volume of the culture medium, which was
 115 colorless/translucent even during I5, was remaining under the B spectrum.

116 All plants survived SGS until I4 (Fig. 1g). The explants were grown under the CW, R2B, and R spectra died
 117 soon after removal from the test tubes. Excessive browning resulted in the death of some explants. At the intersection
 118 of the pseudostem and root, tissue breakage occurred during explant wash, subsequently leading to death, under the R
 119 (1 explant), R2B (1 explant), and CW (2 explants) spectra. No explant loss occurred under the B spectrum.

120 There was a significant interaction between the SPAD index and the light spectrum ($p < 0.0001$). The trends
 121 were similar under the CW, R2B, and B spectra, with a lower index recorded under B; the opposite trends were
 122 observed during I1 and I2, but they remained stable from I3 to I5 (Fig. 1h).

123 Following SGS, all transplanted plantlets survived the acclimatization period. During SGS, light spectrum
 124 affected plantlet height ($p = 0.0181$), leaf number ($p = 0.0053$), and pseudostem diameter ($p < 0.0001$). Plant height
 125 and leaf number increased from I1 to I4. During I5 (Fig. 2d), plants grown under the R and B spectra showed reduced
 126 development. The fewest leaves (Fig. 3a), the shortest plants (Fig. 3b), and the smallest pseudostem diameter (Fig.
 127 3c) were observed under the R and B spectra (Supplementary Figure 3).

128 The present study demonstrated that different light spectra affected all evaluated parameters during *in vitro*
 129 SGS. Regarding plantlet height, there was a reduction from I4 to I5 under all spectra, which was caused by the
 130 shortening of the cigar leaf. In a previous study, the effects of the B and R spectra and their different combinations
 131 during *in vitro* cultivation *Musa paradisiac* ‘Nam Dinh’ were assessed using a sugar-free technique with CO₂ injection
 132 (Nhut et al. 2002). In that study, the plant height was in the order of B < PGF (fluorescent lamp) < R2B (70% red +
 133 30% blue) < R (from the shortest to tallest). These results were similar to those for ‘Prata Catarina’ under 140 days of
 134 SGS in the present study (B<CW<R2B<R) as well as for *H. champneiana* ‘Splash’ under 84 days of *in vitro* culture
 135 under different light spectra (Rodrigues et al. 2018).

136 Similar trends were observed for fresh weight, which was lower under the B spectrum, for ‘Prata Catarina’,
 137 ‘Nam Dinh’, and *H. champneiana* ‘Splash’. Thus, different species exhibited similar behaviors during SGS under
 138 different light spectra. The plantlets were shorter and showed a lower fresh weight under the B spectrum. Leaf number
 139 and explant diameter were not affected by light spectrum during SGS in the present study.

140 In the plantlets, no yellowing or leaf necrosis, a typical symptom of *in vitro* stress caused by the gas-saturated
 141 *in vitro* environment and/or nutrient shortage in the culture medium, was observed (Rodrigues et al. 2018); Pimenta
 142 et al. 2013). In a restricted environment without regular subculture, gas accumulation due to explant growth can be
 143 detrimental to plant development *in vitro*. Ethylene is one of the gases that significantly affect different physiological
 144 processes. Numerous studies have demonstrated the involvement of ethylene in callus proliferation, somatic
 145 embryogenesis, and rhizogenesis (Buddendorf-Joosten and Woltering 1996; Biddington 1992; Marota et al. 2001). In
 146 *H. champneiana* ‘Splash’, leaf senescence was observed under the CW, R2B, and R spectra after 84 days of SGS. The
 147 leaves showed discoloration and necrosis, which are typical symptoms of long-term *in vitro* growth (Rodrigues et al.
 148 2018). In the present study, even under the conditions of restricted gaseous exchange, the ‘Prata Catarina’ explants
 149 were not affected by SGS under different light spectra.

150 Under *in vitro* conditions from I2 to I4, root growth was suppressed under the CW spectrum but compensated
 151 for by the formation of more primary roots. A similar result was observed, albeit to a lesser extent, under the R2B and
 152 R spectra. During the explant wash for data collection, browning of the tissues at the root tips was observed, which
 153 prevented further growth from I3 onward. Meanwhile, this favored an increase in the number of primary roots under
 154 these treatments. During I5, the degree of browning in the roots was higher, which results in the death of some explants
 155 under the CW, R2B, and R spectra. Under the B spectrum, root development was stable, and no symptoms of
 156 senescence were observed until I5. The browning of plant tissues is caused by the oxidation of tannins and polyphenols
 157 and the formation of highly reactive and toxic quinones. This process can start due to successive subcultures, cuts that
 158 generate injuries, or senescence of plant tissues *in vitro* (Ahmad et al. 2013). Marked root senescence during I5 under
 159 the CW, R, and R2B spectra, with the loss of some plantlets, limits the use of these light spectra for 140 days *in vitro*.

160 Plantlets grown under the B spectrum showed greater tolerance of the *in vitro* environment for 140 days,
 161 indicating that a longer period in SGS under this is feasible. The culture medium under the B spectrum remained
 162 translucent without the darkening effect observed in the other treatments. Darkening of the culture medium occurs as
 163 a result of the oxidation of polyphenol exudates from the roots. The absence of this effect under the B spectrum
 164 suggests that blue light, directly or indirectly, inhibits the oxidation of tannins and polyphenols from root exudates *in*
 165 *vitro*.

166 The effect of light quality on the cellular redox balance *in vitro* has been poorly studied and is restricted to
 167 only a few species (Dutta Gupta 2017). A previous study evaluated the antioxidant capacity in *Rehmannia glutinosa*

grown *in vitro* under different light conditions; the highest total antioxidant capacity of the leaf and root extracts was observed in the presence of blue light (Manivannan et al. 2015). At ITC, some banana accessions did not achieve satisfactory development during SGS, and there may be large differences in subculture conditions such as transfer interval, ranging between 3 and 22 months (Panis et al. 2020). The browning effect during *in vitro* banana culture varies among different genomic groups and even within the same subgroup (Van den houwe et al. 1995). The prevention of root browning during SGS under blue light is an important result, which may contribute to controlling browning during banana tissue culture.

The lowest SPAD index was observed from I2 to I5 under the B spectrum, indicating lower photosynthetic activity and, consequently, lower metabolic activity during SGS. These results are similar to those reported previously for 'Nam Dinh', in which blue light completely inhibited plantlet growth (Nhut et al. 2002).

The acclimatized plants showed no morphological variation. In the present study, plants under the B spectrum showed reduced growth during I5 of the acclimatization period. In a previous study, no differences in the height, fresh weight, or SPAD index of acclimatized 'Nam Dinh' plants propagated *in vitro* in PGF under 80% red + 20% blue light were observed (Nhut et al. 2002). The results of the present study are unprecedented and demonstrate that prolonged *in vitro* environmental conditions affect plant development during the acclimatization period.

Factors related to *in vitro* culture techniques, such as stress caused by the accumulation of gases and extreme humidity in the flasks, growth regulator concentrations stimulating bud formation or growth, number of subcultures, and the total duration for which the explant remains *in vitro*, can induce epigenetic alterations or true mutations (Krikorian et al, 1993; Karp 1994; Santos and Rodrigues 2004; Bairu et al. 2006). After acclimatization, the plantlets during I5 under the B spectrum showed normal development, indicating a temporary physiological effect. In the present study, no morphological variations were observed among the evaluated light treatments.

In conclusion, the use of the B spectrum for banana cultivation in a growth chamber or room may be used either exclusively or in combination with other techniques aimed at reducing the growth of the explant without compromising the material quality. This approach may provide more time to resolve operational problems or maintain an active germplasm bank in a CML.

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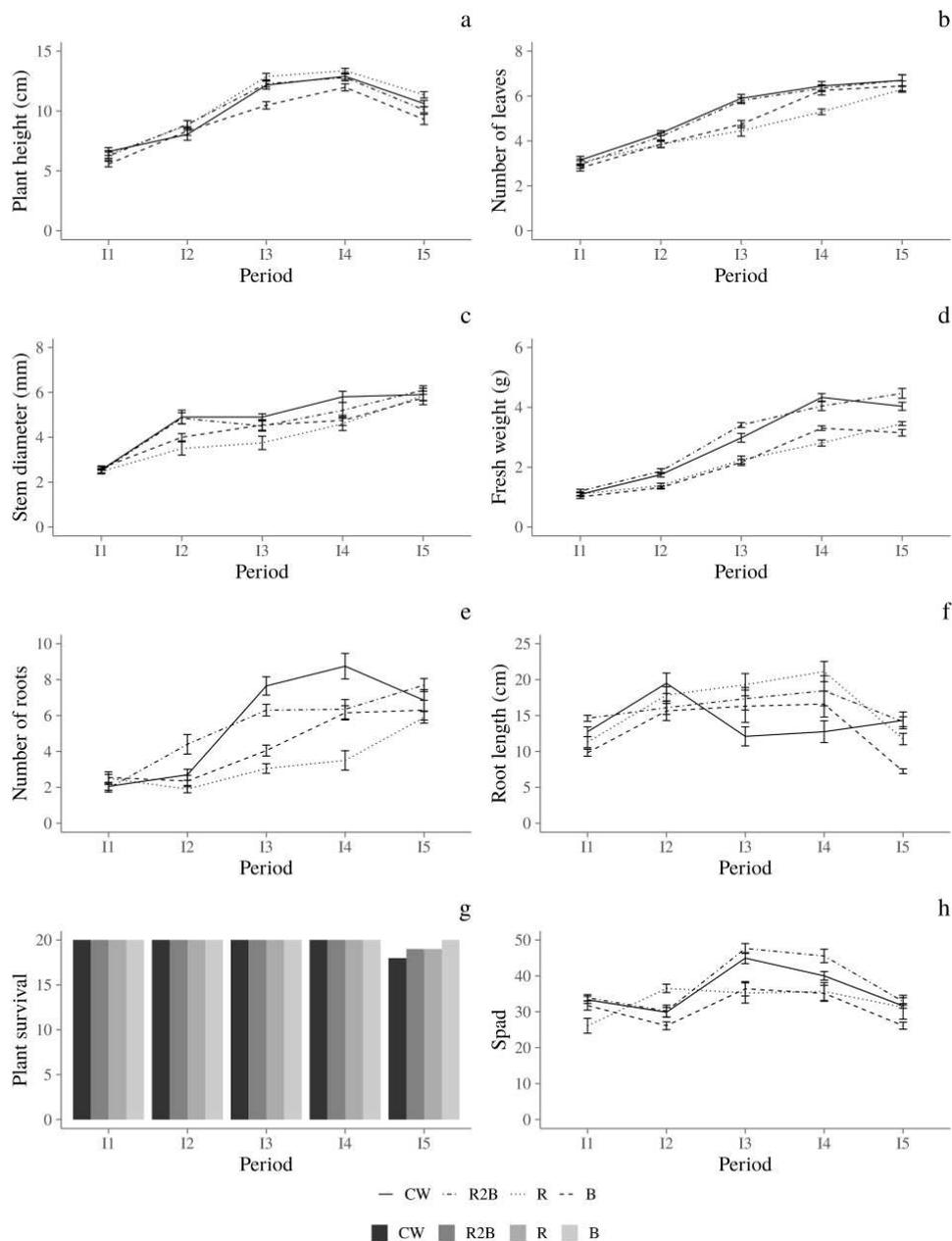
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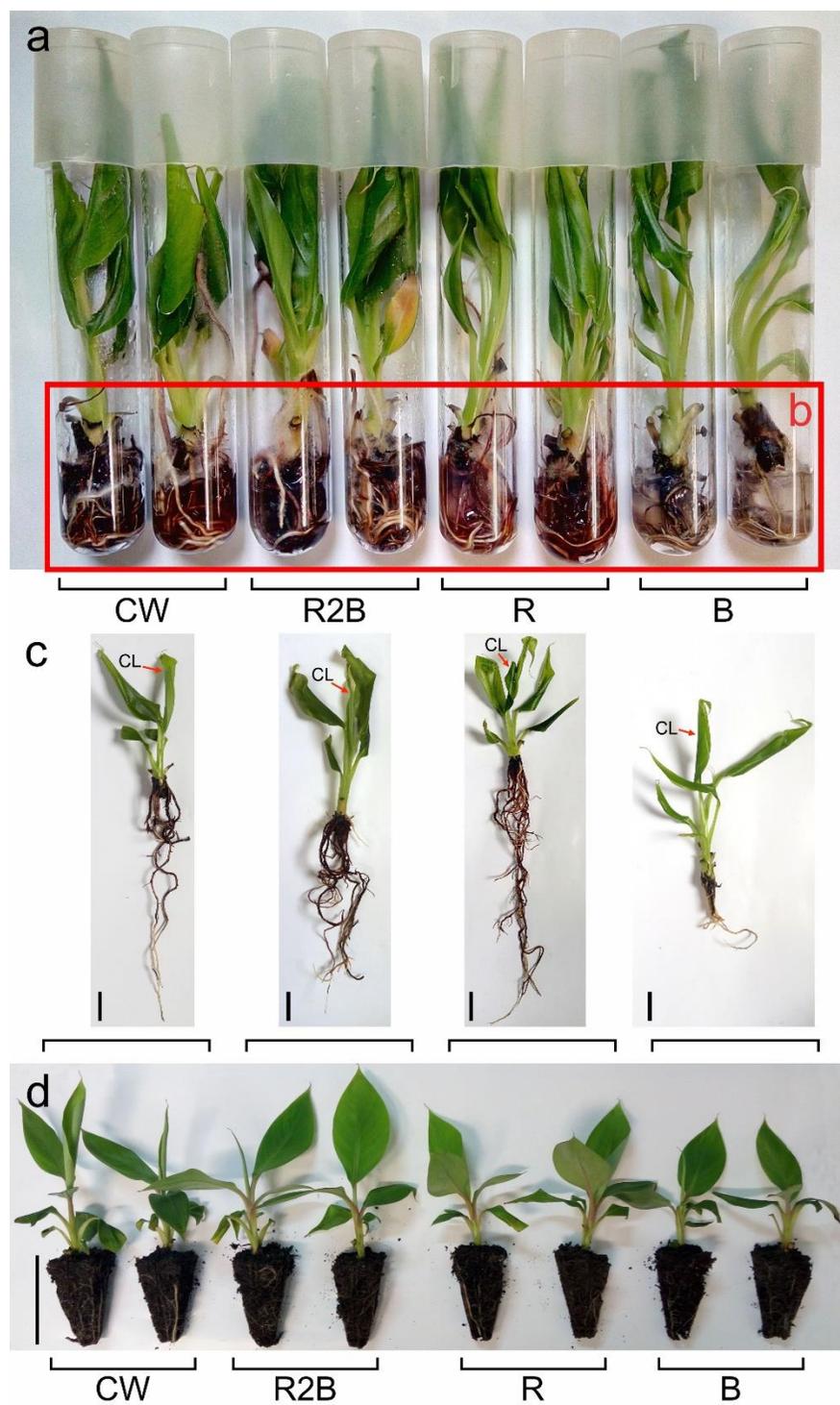
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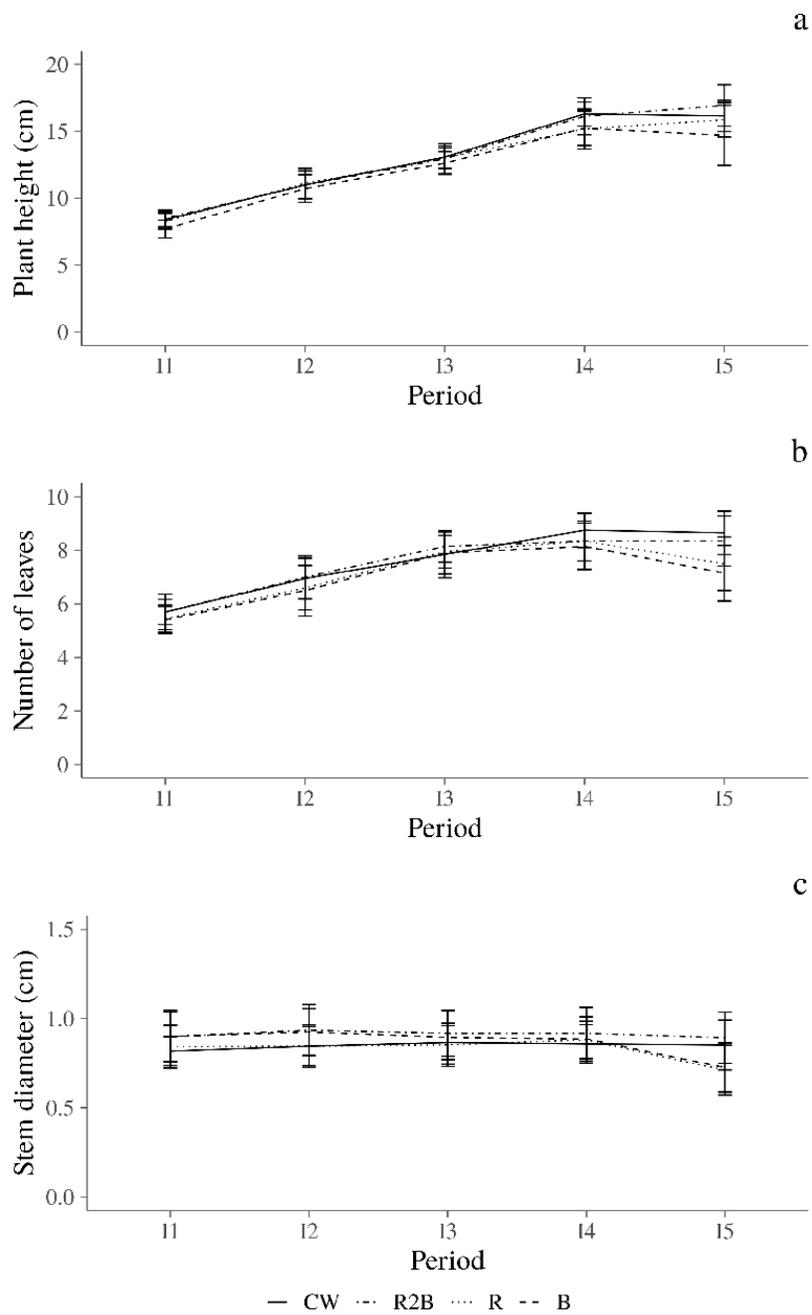
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274 Figure 1- Plantlets in SGS in different LS. Evaluation of banana shoots from cv. Prata Catarina in SGS for
 275 five periods of time in different LS. (a) Plant height-cm, (b) Number of leaves, (c) Stem diameter-mm, (d)
 276 Fresh weight-g, (e) Number of roots, (f) Root length-cm, (g) Plant survival and (h) SPAD. CW (Control
 277 White), R2B (70% Red + 30% Blue), R (100% Red) and B (100% Blue).
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280 Figure 2- (a) Plantlets at different LS in SGS after 140 days (I5). (b) Detail the root plantlets in culture media in I5.
 281 (c) I5 Plantlets before acclimatization. (d) Acclimatized plantlets after four weeks. CL (cigar leaf), CW (Control
 282 White), R2B (70% Red + 30% Blue), R (100% Red) and B (100% Blue). Barr (c) = 1.5 cm, Barr (d) = 6.0 cm



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284 Figure 3- Evaluated banana plantlets cv. Prata Catarina in SGS for five periods after four weeks of acclimatization.

285 (a) Plant height-cm, (b) Number of leaves, (c) Stem diameter-cm.

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