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ESTABLISHING *in vitro* CULTURES *OF Pennisetum* 'VERTIGO®' AND ITS SHOOT MULTIPLICATION UNDER DIFFERENT LED LIGHT QUALITY

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ABSTRACT

This study provides a method for ornamental grass *Pennisetum* 'Vertigo®' *in vitro* culture initiation and shoot multiplication under different LED light qualities. The culture was initiated from field-cultivated plants collected from the 1st to 30th October at weekly intervals. Later dates of collecting shoot tips increased the effectiveness of surface disinfection (from 46% on the first date to 93% on the fifth one) and the percentage of regenerating explants (from 34% on the first date to 93% on the fifth one). Disinfection and regeneration results were better for the apical buds than the axillary buds. Soaking explants in nystatin before surface disinfection or using a medium with an antibiotic increased the effectiveness of disinfection (even by 27–46%, depending on the combination) and did not inhibit the regeneration of explants. At the shoot propagation stage, the multiplication rate was twice as high for the shoots originating from the apical buds (5.5 per explant) than for those originating from the axillary buds. The addition of yellow light to the red and blue light (RBY) spectrum increased the multiplication rate, and the addition of green light to the red and blue (RBG) spectrum increased the fresh weight of the shoots. The highest content of chlorophyll a was found in the shoots propagated under RB, as well as RBY and RBG light.

Key words: Poaceae, micropropagation, ornamental grass, LED

INTRODUCTION

Pennisetum Rich. genus includes about 140 grass species (Poaceae) distributed in tropical and subtropical regions of the world [Ojo et al. 2022] and serve as agriculture and ornamental crops [Guo et al. 2022]. The astounding growth in the popularity of *Pennisetum* species has resulted in breeding programs and the introduction of new cultivars with unique traits that are of interest to the ornamental grass industry [Contreras et al. 2012, Hanna et al. 2011, Hanna and Schwartz 2020]. One of those cultivars is *Pennisetum* 'Vertigo®' registered as Graceful Grasses® Vertigo® *Pennisetum purpureum* 'Tift 8' [https://www.proven-winners.com/plants/pennisetum/graceful-grasses-vertigo-purple-fountain-grass-pennisetum-purpureum]. It is a vegetatively propagated cultivar treated as perennial in mild climates or, most often, as a strong-growing annual plant in areas where it does not overwinter [Hanna et al. 2011]. It is a low-maintenance and high-vigor plant, which, in combination with its large size and unusual blackish purple leaves, makes it a perfect plant for public landscaping and private gardens. The plant height and canopy diameter may vary depending on the season and location, achieving 95–136 cm and 120–159 cm, respectively [Hanna et al. 2016]. Additionally, it is considered to be sterile, and thus, it does not reseed [Hanna et al. 2011]. There is no risk of its invasiveness, which positively distinguishes this cultivar from many other members of this ge-

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nus regarded as an invasive species in many countries [Woods et al. 2012, Reza et al. 2020].

In relation to the above, *Pennisetum* 'Vertigo®' is exclusively a clonally propagated crop. Thus, we suggest *in vitro* micropropagation as a possible way to obtain rapidly multiplying and uniform 'Vertigo®' plant material. *In vitro* propagation protocol can be divided into different stages that should be followed [Abdalla et al. 2022]. In the present work, we focused on the initiation stage, including choosing the type of explant, establishing an aseptic culture, and regenerating shoots from explants cultivated in different light conditions. Recently, various biotechnological approaches have been developed for Poaceae members, in which their shoots [Blinstrubienė et al. 2021, Lal 2021], immature inflorescences [Blinstrubienė et al. 2021, Kopeć and Płażek 2023], seeds [Nuzhyna et al. 2021, Khan et al. 2022], roots [Tiécoura 2003] or leaf sheath explants [Gaikwad and Dobariya 2006] were used for *in vitro* induction of grass cultures. It indicates that the possibilities of obtaining plant propagule are vast in this family, but the details should be refined for specific genotypes. The results (which are often conflicting) of the effects of the artificial light spectrum on the growth and development of plant tissue cultures were extensively described for a large number of species [Cavallaro et al. 2022]. However, there is a lack of a standard protocol including control of light quality for Poaceae species propagated *in vitro* that could support obtaining the plants with desired characteristics. We therefore concluded that the knowledge gap in this critical area of grass micropropagation should be filled.

Scientific recommendations for rooting 'Vertigo®' cuttings via a conventional method supported by a biostimulator have been recently published [Kapczyńska et al. 2020], but micropropagation methods for multiplication of this genotype could be followed throughout the year and used on a large-scale to quickly bring to market a great number of plants that meet high quality and phytosanitary standards, are not available. This paper is the first report to introduce a protocol for the propagation of *Pennisetum* 'Vertigo®' in *in vitro* conditions with relation to the explant origin and light conditions. Our results not only make a significant contribution to the basic knowledge but also allow us to formulate a preliminary protocol that could be used in the commercial production of other ornamental grasses.

MATERIAL AND METHOD

Plant material

To initiate the *in vitro* culture, shoot tips of *Pennisteum* 'Vertigo®' were taken from plants growing in the ground collection (seasonal plants section) of the Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow (50.084292 N, 19.951450 E). The shoot tips were sampled in autumn at weekly intervals (five terms) between October 1st and October 30th. Figure 1 depicts the climatic conditions and dates of the explant collection.

Initiation and stabilization of cultures

The explants were cut from shoot tips (Fig. 2A), with apical buds (Fig. 2B) and axillary buds (Fig. 2C). The list of explants collected on specific dates (with type of disinfection) is presented in Table 1. The explants were rinsed in tap water for 10 minutes and then disinfected in 70% ethyl alcohol for 30 seconds. Subsequently, double surface disinfection was performed using a commercially available preparation (Domestos, Unilever Polska) containing 4.5 g 100 g^{-1} sodium hypochlorite (i.e., 4.28% of active chlorine). First, a 20% concentration of Domestos (Dom) was used for 20 minutes. Next, the explants were rinsed three times in sterile water for 20 seconds, and finally, they were placed in a 2% solution of Dom. Due to infections observed on the first date, the disinfection methods have been expanded, i.e., the explants were additionally soaked in nystatin (at a concentration of 20,000 j.m./mL) for two hours before the disinfection with Dom (N + Dom, Tab. 1) – such treatment was used in the second and fourth term. For the same reason, in the third term, the explants, after surface disinfection with Dom, were placed on a medium enriched with antibiotic Amoxicillin at 150 mg L^{-1} (Dom + A). The antibiotic was added to the medium after cold sterilization with a MILLEX®-GP 0.22 µm syringe filter (Millipore Express® PES Membrane, Merck Millipore Ltd., Ireland). All the applied combinations of apical and axillary bud surface disinfection approaches are shown in Table 1. After the disinfection procedure, the explants were placed individually into tubes with Murashige and Skoog (MS, 1962) basal medium containing 3% sucrose, 5 µM 6-benzylaminopurine (BA), 0.5 µM 1-naphthaleneacetic acid (NAA), solidified with 5% Bioagar (Biocorp, Poland).

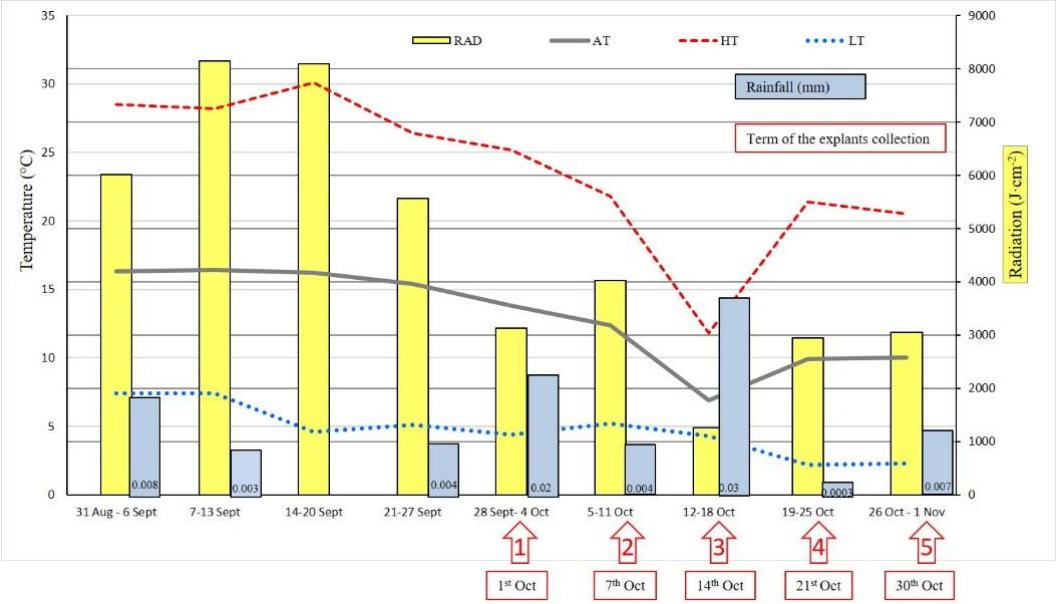

Fig. 1. Weekly average temperature (AT), the lowest temperature (LT), the highest temperature (HT), radiation (RAD), and rainfall during the experiment in the explant collection terms (1–5)

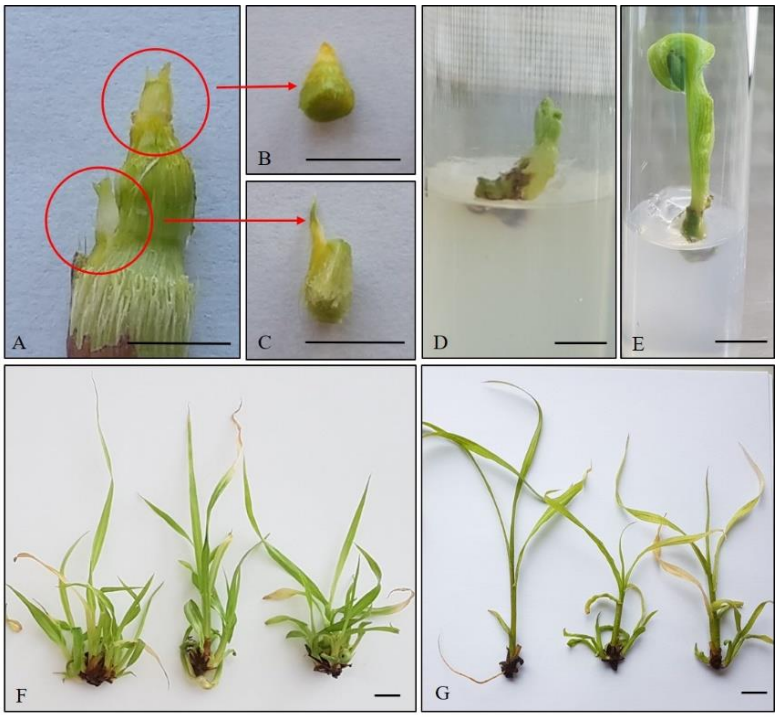

Fig. 2. *Pennisetum* 'Vertigo®' culture initiation (A–E): A – shoot tip for surface disinfection (source of explants), B – apical bud explant, C – axillary bud explant, D – development of an axillary bud (two weeks after the culture initiation), E – development of an apical bud (four weeks after the culture initiation), and shoot multiplication stage (F–G): F – from apical buds and under RB light (after six weeks of the culture), G – from axillary buds and under RB light (after six weeks of the culture); bar = 0.5 cm

Table 1. Methods for surface disinfection of the apical and axillary buds of *Pennisetum* 'Vertigo®' collected in October (five terms) to initiate *in vitro* cultures (Dom – Domestos, N + Dom – nystatin and Domestos, Dom + A – Domestos and antibiotic Amoxicillin in culture medium)

Term	Explant origin		Disinfection		
	apical bud	axillary bud	Dom	N + Dom	Dom + A
1	+	+	+	-	-
2	+	+	+	-	-
	+	+	+	+	-
3	+	-	+	-	-
	+	-	+	-	+
4	+	-	+	-	-
	+	-	+	+	-
5	+	+	+	-	-

Approximately 35–45 explants were cultured per each combination (with three repetitions each). A total of about 240 apical buds and about 160 axillary buds were placed on the initial medium. The cultures were placed in a phytotron, in the dark, at 23°C per 16 h and 21°C per 8 h, and 80% relative humidity. The cultures were observed for ten days for the effectiveness of surface disinfection. The infected cultures were eliminated, and the disinfection rate (i.e., the quotient of disinfected and cultured explants) was calculated. The observations of regenerating explants were conducted after five weeks of culture, and the regeneration rate (i.e., the quotient of regenerated and planted explants) was calculated. The shoots obtained at this stage (Fig. 2E) were multiplied on the same medium for eight weeks (with medium exchange after four weeks). Two initial lines of multiplying plantlets were kept: those derived from the apical buds and those derived from the axillary buds.

Shoot propagation

The plantlets were used to set up the experiment. First, single shoots were separated from them, which were then shortened to a height of 1 cm. The shoots had a four-millimeter fragment of the basal tissue on their base. Five shoots were placed in each 200 mL jar containing a medium of the same composition as during the culture initiation.

In the experiment, four variants of light quality provided with light-emitting diodes (LEDs) (combinations of different wavelengths) were tested: 70% red and 30% blue light (RB), 50% green (λ = 528 nm), 35% red, and 15% blue light (RBG), 50% yellow (λ = 600, 630 nm), 35% red, and 15% blue light (RBY), and white LED light (33% warm 2700 K, 33% neutral 4500 K, 33% cold 5700 K) (WLED). The control conditions were established with white fluorescent lamps (Philips TL-D 36W/54 cool fluorescent lamps) (λ = 390–760 nm) (FL). In total, five different light qualities were tested. The PPFD was 40 μmol m^{-2} s^{-1} in all the combinations. The cultures were kept in a phytotron at 23°C per 16 h of light, 21°C per 8 h of dark, and 80% relative humidity. The cultures involved ten jars per treatment, with five shoots each.

Data collection

Biometrical observations were conducted after six weeks of the experiment to determine the percentage of regenerating shoots, shoot multiplication rate (the number of newly formed shoots per explant), mean shoot length, and fresh weight of shoots. For determination of dry weight, the shoots were dried in a sterilizer (Sanyo Mov 112S, Japan) at 65°C until a constant weight was reached. Photosynthetic pigment levels were determined on a UV/VIS spectrophotometer (Helios Alpha, Unicam Ltd., Cambridge, UK). A 0.2 g plant sample was homogenized in a mortar with a small amount of quartz sand and 4 mL of 96% ethyl alcohol. The resulting mixture was then centrifuged (Eppendorf – Netheler – Hlnz GmbH, 22331 Hamburg) at 10,000 rpm for 15 minutes at 4°C. After centrifugation, 0.5 mL of the extract was taken and mixed with 3.5 mL of ethyl alcohol. Photosynthetic absorption levels were determined at different wavelengths for chlorophyll a (λ = 664 nm), chlorophyll b (λ = 649 nm), and carotenoids (λ = 470 nm) [Sumanta et al. 2014].

Statistical analysis

All collected data were subjected to statistical analysis using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). The effects of the treatments were tested for significance using an analysis of variance (ANOVA). The data were checked for homogeneity of variance. The Duncan post hoc multiple range test was used to separate significantly different means and to provide homogeneous groups for the means (at $P < 0.05$).

RESULTS AND DISCUSSION

Once the ornamental *Pennisetum* hybrids are developed [Hanna et al. 2011, 2016, Hanna and Schwartz 2020], research should begin on developing commercial propagation protocols. Here, we present the results of the first experiments in *Pennisetum* 'Vertigo®' tissue culture. We showed that for the initiation of *in vitro* cultures, the apical buds performed better (higher rate of disinfection and regeneration) than the axillary buds taken from shoot tips (Fig. 3). This was confirmed by the statistical analysis for the Dom-disinfected apical buds at all five dates (Fig. 3A). The effectiveness of surface disinfection was 46–93%, the lowest value was noticed for the explants collected on the first date, and it increased on consecutive dates. Also, the percentage of regenerating explants increased in subsequent terms and ranged from 37 (1st term) to 96% (5th term). A similar trend was observed when analyzing the results for the axillary buds, but both the disinfection and regeneration rates were lower when the axillary buds were used as the explants (Fig. 3B). In the first term, only 4% of the explants were effectively disinfected, but none regenerated. In the fifth term, the disinfection rate was 70%, but less than half of the explants regenerated, which

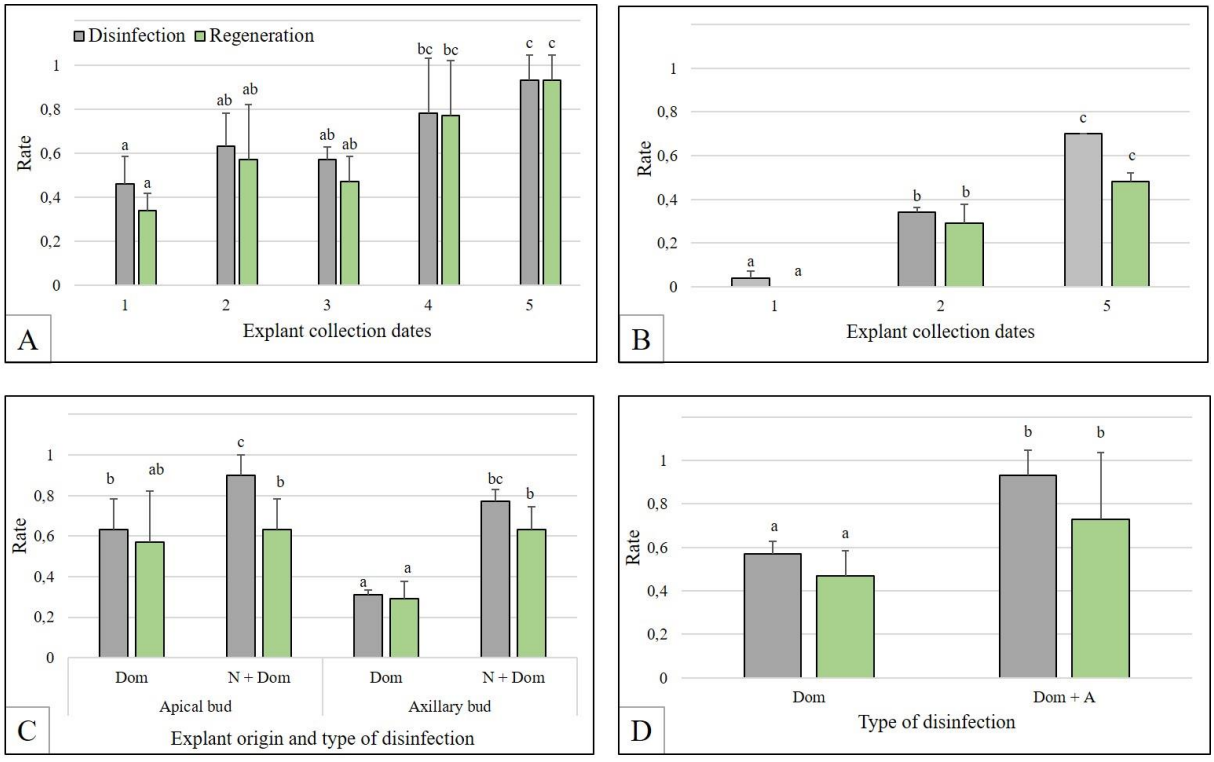

Fig. 3. Disinfection and regeneration of *Pennisetum* 'Vertigo®': A – apical buds collected on five terms (1–5) and disinfected with Domestos (Dom), B – axillary buds collected on three terms (1, 2, and 5) and disinfected with Domestos (Dom), C – apical and axillary buds collected in term 2 disinfected with Domestos (Dom) or with nystatin and Domestos (N + Dom), D – apical buds collected in term 3 disinfected with Domestos (Dom) or with Domestos and cultured in the medium with an antibiotic Amoxicillin (Dom + A); values marked with the same letters do not differ significantly within columns of the same color

Table 2. *Pennisetum* 'Vertigo®' explant regeneration and parameters of the newly formed shoots

Explant origin	Light quality	% of regenerating explants	Multiplication rate	Shoot length (cm)	Shoot fresh weight (mg)	Shoot dry weight (%)
Apical bud	FL	100 ±0.0 c*	5.2 ±1.3 cd	3.0 ±1.2 a	122.2 ±50.2 a	7.7 ±1.3 abc
	WLED	96 ±8.9 bc	4.6 ±0.5 c	4.4 ±0.4 ab	147.0 ±39.9 ab	7.4 ±0.2 ab
	RB	96 ±8.8 bc	5.7 ±0.3 d	3.1 ±0.1 a	115.8 ±12.2 a	8.1 ±0.3 abc
	RBY	100 ±0.0 c	6.8 ±0.3 e	4.0 ±0.5 ab	131.8 ±32.2 a	7.4 ±0.1ab
	RBG	92 ±10.9 abc	5.5 ±0.5 d	3.7 ±0.8 ab	145.2 ±19.0 ab	7.3 ±0.2ab
Axillary bud	FL	76 ±16.7 a	2.7 ±0.3 a	6.0 ±1.5 c	209.3 ±13.8 c	8.5 ±0.5 c
	WLED	92 ±10.9 abc	2.7 ±0.4 a	4.0 ±1.0 ab	163.6 ±35.2 abc	7.9 ±0.4 abc
	RB	76 ±21.9 a	3.0 ±0.5 ab	4.7 ±1.9 b	158.9 ±63.1 ab	8.2 ±0.4 bc
	RBY	80 ±14.1 ab	3.5 ±0.1 b	4.2 ±0.4 ab	188.9 ±3.5 bc	7.6 ±0.4 abc
	RBG	92 ±17.9 abc	2.7 ±0.5a	4.5 ±0.8 ab	209.5 ±31.9 c	7.2 ±0.2 a
Irrespective of light quality						
Apical bud		96.8 ±7.5 b	5.5 ±1.0 b	3.7 ±0.8 a	132.4 ±33.2 a	7.6 ±0.6 a
Axillary bud		83.2 ±17.0 a	2.9 ±0.5 a	4.7 ±1.4 b	186.1 ±39.5 b	7.9 ±0.6 a
Irrespective of explant origin						
	FL	88 ±16.9 a	3.9 ±1.6 ab	4.5 ±2.0 a	165.8 ±57.6 ab	8.1 ±1.0 bc
	WLED	94 ±9.7 a	3.6 ±1.1 a	4.2 ±0.8 a	155.3 ±36.5 ab	7.7 ±0.3 abc
	RB	86 ±18.9 a	4.3 ±1.5 b	3.9 ±1.6 a	137.4 ±48.5 a	8.2 ±0.3 c
	RBY	90 ±14.1 a	5.1 ±1.7 c	4.1 ±0.4 a	160.4 ±37.1 ab	7.5 ±0.3 ab
	RBG	92 ±13.9 a	4.1 ±1.6 ab	4.1 ±0.9 a	177.3 ±42.0 b	7.2 ±0.2 a
Main effects**						
Explant origin		0.0006	< 0.0000	0.0008	< 0.0000	ns
Light quality		ns	< 0.0000	ns	ns	0.0166
Explant origin × Light quality		ns	ns	0.0058	ns	ns

WLED – white LED
LEDs: RB – 70% red + 30% blue; RBY – 50% yellow + 35% red + 15% blue; RBG – 50% green + 35% red + 15% blue; FL – fluorescent lamps
*mean values ± SD in columns followed by different letter(s) are significantly different according to the Duncan's least significant difference test at $p \leq 0.05$
**significant effects ($p \leq 0.05$)
ns – not significant

gives a two times worse result for this date than in the case of the apical buds (Fig. 3A). The observed trend may be due to the fact that the physiological state of the apical buds at shoot collection differed from that of the axillary buds. These differences may be due to the phenomenon of apical dominance, in which apically produced auxin suppresses the axillary buds [Dun et al. 2006], which, as a result, remain in the quiescent phase [Kebrom 2017]. In our experiment, we cultivated the explants on the MS medium containing BA, but only in the basic amount, which could not be enough to promote axillary bud regeneration. Increased levels of endogenous cytokinins would probably release the buds from apical dominance [Müller et al. 2015].

Soaking the explants in nystatin before standard Dom disinfection (N+Dom) significantly improved the efficiency of apical and axillary buds disinfection and also increased the regeneration rate of the axillary buds collected in the second term (Fig. 3C). The addition of Amoxicillin (Dom + A) to the medium at a concentration 150 mg/1000 ml, carried out for the apical buds collected in the third term, significantly increased the disinfection rate (from 57 to 93%), and also the regeneration rate from 47 to 73% (Fig. 3D). This indicated that sodium hypochlorite (Dom), which is most often used for surface disinfection of plant material [Barampuram et al. 2014], was not enough to efficiently remove contaminants from *Pennisetum* 'Vertigo®' explants, espe-

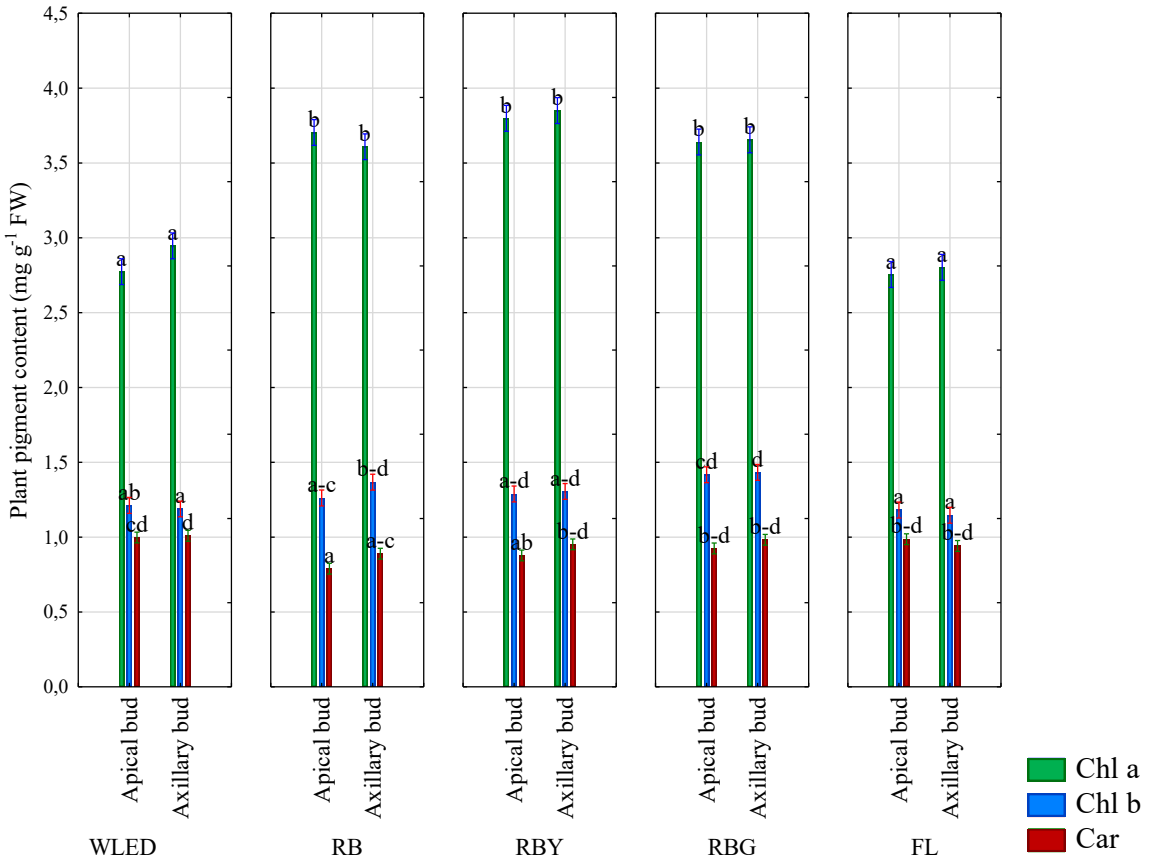

Fig. 4. Effect of explant origin (apical or axillary bud) and light quality on the plant pigment content in *Pennisetum* 'Vertigo®' shoots (WLED – white LED; LEDs: RB – 70% red + 30% blue, RBG – 50% green + 35% red + 15% blue, RBY – 50% yellow + 35% red + 15% blue; FL – fluorescent lamps)

cially for the first batches of the culture establishment. We observed contamination that indicated the presence of pathogens and could not be eliminated during disinfection with Dom. Therefore, in the second term, we tested an antifungal agent, nystatin, to improve the disinfection protocol. In the third term, we also added Amoxicillin to investigate the effect of antibiotics on the culture of this grass. Our results (more effective disinfection and regeneration of the explants than when using Dom alone) confirmed the correct selection of these agents. Most importantly, these agents, while destroying pathogens, did not reduce the regenerative potential of the explants. However, in order not to incur additional costs related to the decontamination of the initial material, we recommend collecting the explants at later terms because, as our results showed, the effectiveness of Dom increased at later terms of the explant collection. All successfully disinfected apical buds from the fifth batch started to grow (Fig. 3A).

The shoots grown from the apical buds had a higher regenerative potential, as almost all of them regenerated (Tab. 2). Contrary to that, 8–24% of the shoots derived from the axillary buds died. The same trend was noted for the multiplication rate. Almost two times more new shoots developed from the apical buds (4.6–6.8) than from the axillary buds (2.7–3.5). Again, this can be explained by the fact that the axillary buds are in a different physiological state (activation level) than the apical buds, and their growth potential depends on the inhibitory effect of parent apical buds [Thomas and Hay 2009]. Most shoots were observed under yellow-enriched RB light (RBY). The shoots from the axillary

buds were longer than those from the apical buds, and the most extended shoot was obtained under fluorescent light (6 cm). The length of the shoots, depending on the experimental combination, was 3–6 cm. A similar relationship was observed for the fresh weight of shoots. Those from the axillary buds were 40% heavier than those obtained from the apical buds.

The origin of the shoots had no effect on dry weight, but this trait was affected by the quality of the light (Tab. 2). Irrespective of the explant origin, the highest dry weight (8.2%) was determined for the shoots propagated under RB light. It was not significantly different from that recorded for the shoots grown under the control fluorescent lamp and white LED light (8.1 and 7.7%, respectively) (Tab. 2).

The light spectrum under which the shoots were cultivated determined the content of photosynthetic pigments (Fig. 4). All the explants contained more chlorophyll a under RB light, and also when RB light was enriched with yellow or green light (3.608–3.849 mg g^{-1} FW), than under the combination with white LED and fluorescent light (2.744–2.947 mg g^{-1} FW). When evaluating the content of chlorophyll b, a similar relationship was observed, and the highest value of this pigment was determined in the shoots propagated under RBG light (1.417–1.433 mg g^{-1} FW). Our findings showed that the combination of RB LED lighting systems, as well as the combination with green and yellow LED light, are more suitable for chlorophyll synthesis by *Pennisetum* 'Vertigo®' shoots than white LED or fluorescent lamps. For this reason, fluorescent lamps are being slowly replaced with light-emitting diodes that are more durable, do not require regular replacement, and generate far less heat in a growth room [Bello-Bello et al. 2017].

The contents of carotenoids were lower than those of chlorophylls, but we did not find a clear pattern of response in the shoots cultivated under different light conditions in relation to carotenoid production. Carotenoid content in the shoots was at the level of 0.790–1.010 mg g^{-1} FW and depended on the light quality (Fig. 4). These values were higher than those reported for seedlings of *Pennisetum glaucum* (0.2 mg g^{-1} FW) grown in laboratory conditions under luminescent white light tubes [Toderich et al. 2018], or lower than for *Pennisetum typhoides* cultivated in the field (1.202 mg g^{-1} FW) [Rajput and Patil 2017]. It indicates that the accumulation and composition of plant pigments in different genotypes representing the same genus may differ in relation to the intensity and composition of light under which they are grown.

CONCLUSIONS

During the initiation of *Pennisetum* 'Vertigo®' cultures in autumn, the later term of shoot tip collection increased the effectiveness of surface disinfection and the number of regenerating explants. At the last initiation term, almost 100% of the apical buds used as explants were successfully disinfected and regenerated. The apical buds always reached higher disinfection and regeneration rates than the axillary buds used as explants. Only in the case of culture initiation at earlier terms, we recommend soaking the explants in nystatin before surface disinfection or using a medium with an antibiotic, as these agents increased the effectiveness of disinfection and did not inhibit the regeneration of explants. At the shoot propagation stage, the multiplication rate was twice as high when the shoots originated from the apical buds as compared with those that originated from the axillary buds. The addition of yellow light to the spectrum of RB light increased the multiplication rate, and the addition of green light to the spectrum of RB increased the shoot fresh weight. The highest dry weight was found in the shoots cultivated under RB light. The shoots propagated under RB, RBY, and RBG light had the highest content of chlorophyll a, similar to chlorophyll b, but the latter pigment was the most abundant in the shoots developed under RBG light.

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