An Efficient Method of *Pennisetum x Advena* 'Rubrum' Seedlings Production Using Temporary Immersion Bioreactor and Agar Cultures

Mariusz Pożoga (✉ mariuszpozoga@gmail.com)

Warsaw Agricultural University: Szkoła Główna Gospodarstwa Wiejskiego w Warszawie

Dawid Olewnicki

Warsaw University of Life Sciences: Szkoła Główna Gospodarstwa Wiejskiego w Warszawie

Elżbieta Wójcik-Gront

Warsaw University of Life Sciences: Szkoła Główna Gospodarstwa Wiejskiego w Warszawie

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Research Article

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Abstract

The aim of this study was to propose an efficient method of *Pennisetum x advena* 'Rubrum' micropropagation. Agar cultures with MS medium supplemented with BAP in various concentrations (0.5 mg/L-2 mg/L) and a temporary immersion bioreactor system (TIS) with liquid medium MS with an addition of 1 mg/L BAP were used. For rooting ½ MS medium with different auxin combinations (IBA, NAA) and activated charcoal was utilized. The most efficient method turned out to be TIS which produced 36.9 new plants in four weeks. The seedlings were slender in shape, bright green in colour with no signs of hyperhydricity. The most suitable agar medium produced 19.5 new plants in an eight week period. Rooting should be carried on ½ MS supplemented with 0.5 mg/L IBA and 0.5 mg/L NAA with an 84% rooting rate. The addition of activated charcoal inhibited rooting.

Key Message

The aim of the study was to examine the most efficient micropropagation method of *Pennisetum x advena* 'Rubrum' in agar cultures and its comparison to temporary immersion bioreactor system performance.

Introduction

Pennisetum x advena 'Rubrum', also known as *Pennisetum setaceum* 'Rubrum' or purple fountain grass is a plant with great commercial potential (Yue et al., 2020). *Pennisetum x advena* is considered a cross between *Pennisetum setaceum*, from North Africa, and *Pennisetum Macrostachys* from Malesia (Wipf and Veldkamp 1999; Padhye et al. 2008). *Pennisetum x advena* 'Rubrum' is a triploid ($2n=3x=27$). It is rather sterile and its seed production is extremely low (Simpson and Bashaw 1969; Zhang et al. 2015). It belongs to the *Poaceae* family. There are more than 80 known *Pennisetum* species in the *Poaceae* family. It requires little care, not demanding, drought resistant but sensitive to low temperatures (Contreras et. al, 2013).

In vitro plant propagation is an advanced technology, which enjoys growing interest due to the high demand for genetically identical plants of excellent quality. This technique allows for efficiently multiplying disease-free plant material. The history of *in vitro* cultures dates back to the beginning of the 20th century, when in 1902 the German physiologist Gottlieb Haberlandt proposed creating a cell culture in artificial conditions. He isolated single cells from various plant species and was the first to make them grow. However, he failed to cause cell divisions (Haberlandt, 1902). Haberlandt is called the father of tissue cultures thanks to his work and his thesis on the factors influencing *in vitro* culture. The next important event was F.W. Went's auxin isolation in 1928 (Masuda and Kamisaka, 2000). In 1962 Murashige and Skoog (Murashige and Skoog, 1962) prepared macro- and micronutrients composition called MS medium which is commonly used nowadays. As a result, micropropagation techniques began to be commercialised and used in mass plant propagation very quickly. Besides micropropagation *in vitro* technology is used for obtaining new varieties by polyploidization (Escandón et al 2006) or doubled

haploids (Chaikam et al. 2020), preserving threatened species (Sarasan et al. 2006), virus eradication (Wang et al. 2018) and producing bioactive compounds by hairy roots (Kareem et al. 2019; Kowalczyk et al. 2020).

The increase in competition on the plant market has forced the search for new, better solutions in plant production which permit lowering production costs. Bioreactors can provide such solutions. Over the years many kinds of bioreactors have been developed, including: stirred tank bioreactor, cone balloon-type airlift bioreactor, temporary immersion system TIS (systems: SETIS and RITA), rotating drum bioreactor, nutrient mist bioreactor, radial flow bioreactor or wave bioreactor (Takayama and Akita 1994; Steingroewer et al. 2013; Werner et al. 2018). The most popular TIS use a liquid medium. As agar is the most expensive ingredient of tissue culture medium it is great advantage (Pożoga et al. 2019). Moreover liquid cultures provide more uniform culturing conditions and medium exchange without container change and plant passaging is reduced. Gas exchange, which accelerates growth, is also very advantageous (Etienne and Berthouly 2002; Murch et al. 2004; Albarran et al. 2005).

Materials And Methods

The plant used in the experiment was *Pennisetum x advena* 'Rubrum'. The explant for culture initiation was 1 cm intercalary meristem. The explants were surface sterilized with 15% commercial bleach solution (4,28% sodium hypochlorite) for 15 minutes. Afterwards it was rinsed with sterile distilled water three times for 5, 10 and 15 minutes respectively. The medium used for culture initiation was Murashige and Skoog MS (Murashige and Skoog 1962) medium with vitamins: 2 mg/l Glycine, 100 mg/l myo-Inositol, 0.5 mg/l Nicotinic acid, 0.5 mg/l Pyridoxine, 0.1 mg/l Thiamine supplemented with 1 mg/l 6-benzylaminopurine (BAP). To find the most efficient medium for multiplication MS medium with vitamins was used with the following BAP concentrations: 0,5 mg/l (M1); 1 mg/l (M2); 1,5 mg/l (M3) and 2 mg/l (M4). MS medium with vitamins and 1 mg/l BAP (M5) was used for the temporary immersion bioreactor system. For rooting half strength MS medium with ½ vitamins (R1) was used and its combination supplemented with 2% activated charcoal (RAC1); half-strength MS medium with ½ vitamins and 0,5 mg/l IBA (R2); half-strength MS medium with ½ vitamins, 0,5mg/l IBA and 2% activated charcoal (RAC2); half-strength MS medium with ½ vitamins, 0,5 mg/l IBA and 0,5 mg/l NAA (R3); half-strength MS medium with ½ vitamins, 0,5 mg/l IBA, 0,5 mg/l NAA and 2% activated charcoal (RAC3). Every medium was supplemented with 2% sucrose and 7 g/L plant agar besides the bioreactor combination M5. The pH was adjusted to 5.8 before autoclaving. The photoperiod consisted of 12 hours of daylight and 12 hours of night breaks with lighting provided by cool white fluorescent tubes (3100 lm) at a stable temperature of 23 °C during the day and night. The subculture time in agar cultures was eight weeks and four weeks in TIS. The agar cultures were conducted in 350 ml plastic containers with ten explants in each. The temporary immersion bioreactor was built with two 1.8 L jars. In medium supplying a 400ml jar of medium was used. Ten explants were placed in the second jar. The immersion frequency was 1min/1h. Quantity observations were conducted after eight weeks in the case of multiplication in agar cultures,

four weeks in TIS and three weeks for rooting. The experiment was repeated three times. Each time there were ten containers with ten explants. The plants were acclimatised to greenhouse conditions.

The results of the above experimental assumptions were analyzed statistically. All of the statistical calculations were performed using the STATISTICA data analysis software, version 13.0 (TIBCO StatSoft Inc. StatSoft Poland, Warsaw). To determine whether the sample distribution matches the characteristics of a normal distribution, the Kolmogorov-Smirnov test was used. Afterwards, the non-parametric Kruskal-Wallis test was used to evaluate the differences between the tested methods. For all of the calculations, the statistical significance was set at $p < 0.05$.

Results

In agar mediums the best multiplication rate after eight weeks was achieved on medium M2 and M3 with an average number of new plants 19.5 and 18.1 respectively (Fig. 1B, Fig. 1C). The minimal number of new plants in M2 was seven and the maximal number amounted to 34 new plants. In M3, the minimal number of new plants was 5 and the maximal number of new plants was 31. M1 and M4 resulted in a much lower multiplication. M1 medium generated 12.7 new plants with a minimal number of four new plants and a maximal number of 22 new plants (Fig. 1A). M4 medium generated 12.9 new plants where the minimal number of new plants was three and the maximal number of new plants was 24 (Fig. 1D). The plants obtained in medium M1, M2 and M3 were slender in shape and green in colour (Fig. 1F). The plants grown on M4 were very hard, slightly vitrified, extended at the base, and the leaves were slightly red at the end (Fig. 1G). The highest number of plants was achieved in a bioreactor in M5 and this method gave significantly different results from the others. The mean number of new plants after four weeks was 36.9 per explants. The minimal number of new plants was 15 and the maximal number of new plants was 55. Means for all combinations, medians and minimums and maximums are presented in Fig. 2.

The morphology of new plants was the same as in M1-M3. TIS and plants are presented in Fig. 1E and Fig. 1H. Taking into account the results of statistical analyses it can be concluded that the sample distributions did not match the characteristics of a normal distribution. Thus, according to Kruskal-Wallis, the methods differed significantly from each other ($p < 0.05$). Multiple sample comparisons indicated that the M1 and M4 mediums were not significantly different, as were the M2 and M3 mediums.

The rooting rate was established after three weeks. Medium R3 turned out to be the most efficient, with 84% plants rooted. The minimal number of rooted plants was five out of ten and the maximal number of rooted plants was ten. The roots were long, unbranched and healthy (Fig. 3C). Only 36% of plants were rooted on R2 and only 12% on R1. The minimal number of rooted plants on R2 and R1 was two and zero, and the maximal number of rooted plants was six and three respectively. The roots developed on R2 were shorter than on R3 and were slightly branched (Fig. 3B). The plants on R1 had very short, unbranched roots (Fig. 3A). The media containing activated charcoal showed strong inhibition in rhizogenesis. Medium RAC1, RAC2 and RAC3 showed no roots (Tab 1). The rooted plants are presented in Fig. 3D.

In terms of rooting results the sample distributions also did not match the characteristics of a normal distribution. Thus, according to Kruskal-Wallis, the methods differed significantly from each other ($p < 0.05$). Multiple sample comparisons indicated that all of the methods were significantly different (Tab 1).

The next stage of the experiment included transferring plants to multi-pots and acclimatising to greenhouse conditions. At this stage of the experiment, 100% of the plants survived and started rapid growth (Fig. 3D).

Discussion

As *Pennisetum x advena* 'Rubrum' is an ornamental plant with considerable market value, an efficient propagation method is essential. The examined mediums in our study showed various effects with significant differences. When using common agar culture the highest number of new plants was achieved in the MS medium containing 1 mg/l BAP. Wei et al. (2015) indicated that the most optimal medium for *Pennisetum x advena* 'Rubrum' micropropagation was MS with an addition of 1.5 mg/l BAP, 0.1 mg/l NAA and 0.5 mg/l IBA. The multiplication coefficient in this medium reached 6.5 after 30 days. In our experiment on MS medium supplemented with 1,5 mg/l BAP with no auxin addition we obtained 18.1 new plants after eight weeks. This may suggest that besides medium composition passage time is essential in *Pennisetum x advena* 'Rubrum' micropropagation. Elongated passage time on a similar medium ensured a result approximately three times better. The use of a very high BAP level in *Pennisetum* micropropagation also can generate a large number of new plants. MS with 4 mg/l of BAP allowed for obtaining 26,6 new shoots of *Pennisetum glaucum* after 30 days (Maity et al. 2009). The same author also used callus regeneration. The results were dependent on the explant type and genotype. The best results of 10.2 plants per callus after 30 days were achieved on MS with an addition of 2 mg/l BAP. Yue et al. (2020) proposed a method of micropropagation of triploid and hexaploid *Pennisetum x advena* with callus induction on MS with an addition of 3 mg/l 2,4 – D, 1 mg/l NAA, 1 mg/l KIN in the dark. Next cultures were transferred on MS with 3 mg/l BAP and 0.5 mg/l NAA for shoot induction for 4-6 weeks and then transferred to ½ MS rooting medium. Some reports of other *Pennisetum* species micropropagation protocols via callus indicate the popularity of these methods: *Pennisetum americanum* (Plus et al., 1993) or *Pennisetum glaucum* (Mythili et al., 1997; Lambé et al. 1998).

The rooting percentage of *Pennisetum x advena* 'Rubrum' was the highest in ½ MS with an addition of 0,5 mg/l IBA and 0,5 mg/l NAA and it was 84%. Wei et al. (2015) on the same medium achieved 100% rooted plants. Differences can be explained by using different genotypes. Many investigators suggest that activated charcoal improves rooting (Dumas and Monteuis, 1995; Sanchez et al., 1996; Oakes et al. 2021). In our study adding of activated charcoal to any medium inhibited rooting. This could be explained by auxin adsorption by activated charcoal.

There are no reports about bioreactor propagation of *Pennisetum*. Temporary Immersion Bioreactors (TIS) seem to be the most perspective for commercial tissue culture laboratories because of compact size and ease of use. Moreover a great advantage of TIS is: its ability to reduce of hyperhydricity in

comparison to permanent immersion (Afreen, 2006). In our experiment hyperhydricity was not observed. We achieved 90% more new plants in TIS than in agar culture twice as quickly. Many researchers have also indicated the better performance of TIS over standard agar-based cultures. Murch et al. (2004) demonstrated a five times bigger fresh weight per plantlet of *Crescentia cujete* and two times better rooting rate. Businge et al. (2017) observed an approximately 100% higher multiplication rate, 60% more fresh weight of *Betula pendula* and a 500% higher multiplication rate, and an 1100% more fresh weight of *Eucalyptus* species. Uma et al. (2021) indicates that the multiplication of new banana plant shoots in TIS was 2,7 higher than the semisolid culture method. Moreover Yan et al. (2010) and Jiménez et al. (1999) indicated higher multiplication of plants in TIS prior to agar cultures. The temporary immersion bioreactor method shows much higher efficiency than agar cultures therefore further investigation and method improvements should be developed.

Conclusions

1. When using temporary immersion system in *Pennisetum x advena* 'Rubrum' micropropagation, 90% more new plants can be obtained in comparison to agar cultures in two times shorter time with no signs of hyperhydricity.
2. The most efficient medium occurred to be MS medium with an addition of 1 mg/l BAP in agar and TIS combinations. The TIS method gave statistically different results from the others.
3. Rooting should be provided on ½ MS supplemented with 0.5 mg/l IBA and 0.5 mg/l NAA.
4. The addition of activated charcoal inhibits rooting.

Declarations

Author Contributions Conceptualization, methodology, writing—review and editing was performed by M.P., D.O. and E.W.G.; writing—original draft preparation, validation, formal analysis, investigation, visualization was executed by M.P.; data curation was performed by E.W.G.; supervision and project administration was performed by D.O.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not require any study with humans or animals

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Tables

Table 1. Descriptive statistics (percentage. maximum. minimum. Q1 - the first quartile. median. Q3– the third quartile) of the rooted plants in each medium. . Multiple comparisons of used rooting mediums. Significantly different methods were marked in *.* p < 0.05

Medium	Specification and multiple samlpe comparisons								
	percentage	minimum	maximum	Q1	median	Q3	R1	R2	R3
R1	12	0.0	3.0	0.0	1.0	2.0		*	*
R2	36	2.0	6.0	3.0	3.0	5.0			*
R3	84	5.0	10.0	8.0	8.5	9.0			

Figures

Figure 1

Effect of multiplication mediums. (A). Plants obtained after 8 weeks on M1. (B). Plants obtained after 8 weeks on M2. (C). Plants obtained after 8 weeks on M3. (D). Plants obtained after 8 weeks on M4. (E). Plants obtained after 4 weeks on M5. (F). Representative single plant from M1, M2, M3, M5. (G). Representative single plant from M4. (H). Temporary immersion bioreactor system.

Figure 2

A box plot with the first and third quartiles, the median as well as minimums and maximums depicted with whiskers for number of newly emerged plants in each method.

Figure 3

Effect of rooting medium and acclimatisation. (A). Roots obtained on R1 after 3 weeks. (B). Roots obtained on R2 after 3 weeks. (C). Roots obtained on R3 after 3 weeks. (D). Acclimatised plant.

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