

Effects of Polyvinyl Pyrrolidone and Activated Charcoal to Control Effect of Phenolic Oxidation on *In Vitro* Culture Establishment Stage of Micropropagation of Sugarcane (*Saccharum Officinarum L.*)

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Abstract

Sugarcane is grown in Ethiopia as both cash and industrial crop. Although it plays a great role in the economy of the country, there are not enough sugarcane varieties under production and it is not an ideal crop for conventional breeding. Hence, it takes around ten years for its breeding and extra years to scale up the planting material for commercial by vegetative propagation. To circumvent these limitations, biotechnological tool (Plant tissue culture) was born as best alternative. Though the tissue culture (micro propagation) plays a reliable role, culture establishment stage of sugarcane micro propagation has a serious phenolic oxidation problem which can kill the whole culture. Hence, these experiments were conducted to optimize the appropriate concentration of Polyvinylpyrrolidone (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 gL⁻¹) and Activated charcoal (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 gL⁻¹) on (C86-12, C86-56) genotypes in completely randomized design with 2 × 5 factorial treatment combinations arrangements to control the effect of phenolic oxidation. Analysis of variance showed that interaction effect of Polyvinylpyrrolidone and genotypes. Activated charcoal and genotypes on percentage of survived and dead explants due to phenolic oxidation were highly significant at (P=0.001). Murashige and Skoog medium supplemented with 0.2 gL⁻¹ and 0.3 gL⁻¹ of Polyvinylpyrrolidone has gave 100% and 80% survived explants of C86-56 and C86-12 genotypes respectively while 0.4 gL⁻¹ and 0.3 gL⁻¹ of activated charcoal resulted in 46% and 40% of survived explants of C86-56 and C86-12 genotypes respectively, after 30 days of culturing. Thus, these optimized concentrations of Polyvinylpyrrolidone and activated charcoal are useful to control the effect of phenolic oxidation on culture establishment of micro propagation, which helps to have enough starter culture for further multiplication stage of micro propagation.

Keywords: Sugarcane; Phenolic oxidation; Polyvinylpyrrolidone; Activated charcoal; Survived explant

Introduction

Sugarcane (*Saccharum officinarum L.*) belongs to the *Saccharum* genus of the Andropogoneae tribe of the Poaceae (Gramineae/grass) family with an octaploid 2n=8 × =80 number of chromosomes [1]. It is a perennial cash crop grown in tropical and subtropical regions of the world, which accounts about 75% of the sugar in the world [2]. The commercially cultivated crops of sugarcane have two geographic centers of origin; in Newguinea and Northern India [3]. It is an important cash crop and the main source of raw material for the production of sugar [4]. It is cultivated as a commercial crop in nearly around 60 countries [5]. In Ethiopia sugarcane is grown both as cash and industrial crop but there is no well documented reference on how, where and when it was introduced, although some records claim its introduction during the early 18th century [6]. Its properties such as efficient photosynthesis and biomass production make it an excellent target for industrial processing, valuable alternative for animal feed and production of bi-products such as ethanol production from molasses [7]. In Ethiopia the annual yield of sugar was around 300,000 tons from three established sugar factories but the annual domestic demand close to 450,000 tons [8]. However, the country has abundant water resource coupled with a vast fertile land favorable for sugarcane cultivation, suitable agro-ecological conditions, cheap labour and huge domestic and foreign demand for sugar and its by-products. So, by realizing these potentials and opportunities, the Ethiopian government has planned to establish ten sugar factories with 370,000 ha of plantation area.

Though, the edaphic and climatic factors are suitable for sugarcane cultivation, there are limited varieties under production and the country

has no breeding facilities to release new high yielding and disease resistance varieties. Furthermore sugarcane is not an ideal candidate crop for conventional plant breeding; because its flowering is not-synchronised, it has low sexual seed viability and it is a perennial crop [9]. Hence, its improvement takes up to ten years from initial crosses to final agronomic assessment of elite varieties [10,3]. In addition its vegetative propagation by cutting for commercial production takes extra years and favors dissemination of disease and pest from generation to generation [11,12]. To circumvent these limitations of conventional breeding and vegetative propagation of sugarcane, biotechnological tool (plant tissue culture) was born as best alternative.

So far in Ethiopia, new varieties have been imported and propagated vegetatively by cutting for commercial production. But currently, after importing the new varieties, to supplement the vegetative propagation, we have already started *in vitro* propagation of sugarcane varieties. Though sugarcane tissue culture is the best alternative technology

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or option, its operation is very difficult; because sugarcane has high content of phenols, especially in the apical meristem and leaf sheaths which poses browning problem during tissue culture process [13]. It is a serious problem associated with the culture establishment stage of micro propagation and the oxidation of phenolic substances leaches out from the explant resulting in browning of the explant, the medium and at last killing the whole culture [14]. Pretreatment of the explants with solutions of ascorbic acid, citric acid, Polyvinylpyrrolidone (PVP) and activated charcoal (AC) or culturing them with these antioxidants could solve the problem [14]. By using 0.3 gL⁻¹ PVP [15] Shukla RK et al. found that all their cultures were survived. Around 80% browning free cultures (explants) were obtained by using activated charcoal of 0.2-0.5 gL⁻¹ [16]. Hence, this research was conducted to optimize appropriate concentrations of PVP and AC to control the effect of phenolic oxidation on culture establishment stage of micro propagation which helps to have enough starter culture for the subsequent multiplication stage of micro propagation.

Materials and Method

The experiment was done at Plant tissue culture laboratory of Jimma University College of Agriculture and Veterinary Medicine (JUCAVM), Ethiopia. Two sugarcane genotypes, (C86-12 and C86-56), were used for the study. They were imported from Cuba in 2006. After agronomic performance evaluation; these genotypes were among the selected ones to be commercialized. The sets of these genotypes were prepared, treated with hot water and taken to JUCAVM green house and planted. After two to three months of growing, shoot tip explants were taken from the sugarcane plants. The explants were prepared according to Jalaja NC et al. procedures [12]. The surrounding leaf sheaths of sugarcane tops were carefully removed one by one until the inner white sheaths were exposed. The explants were sized to about 10 cm length by cutting off at the two ends, locating the growing point somewhere near to top. They were washed under running tap water and liquid detergents. They were soaked in fungicide solution (0.3% kocide) for 30 minutes under laminar flow cabinet containing three drops of tween-20. After the kocide was properly washed off from the explants, they were rinsed three times with distilled water and disinfected with 70% ethanol for one minute. The ethanol was poured off and the explants were rinsed again with sterile distilled water. Disinfection of explants was done with 0.1% of HgCl₂ for 10 minutes [17] followed by 3-4 washing with sterile distilled water. The required amounts of all stock solutions of MS [18] medium, 20 gL⁻¹ sucrose [19], different concentrations of PVP and AC and 3 mgL⁻¹ of BAP [20] were mixed in a beaker and the pH was adjusted to be 5.8. This was followed by addition of 0.8% agar for solidifying the media. Then, it was heated to melt the agar and then 30 ml media was dispensed in to culture jars. Finally, it was autoclaved at temperature of 121°C for 20 minutes with 15 psi of pressure.

About 1.5 cm explants were cultured under laminar flow hood aseptically and transferred to the growth room at which growth conditions were adjusted to be 16 hr of light and 8hr of dark with 25 μmolm⁻²s⁻¹ photosynthetic photon flux intensity and 26 ± 2°C of temperature. The experiments were laid down in complete randomized design with two factor factorial treatment combinations arrangements and each treatment was replicated three times. Data of survival and death of explants due to phenolic oxidation (browning) were collected after 30 days of culturing and subjected to two-way analysis of variance (ANOVA) using SAS statistical software version 9.2 (SAS Inc., 2008). Treatments means were separated by using REGWQ (Ryan-Einot-Gabriel-Welsch Multiple range test) mean separation method.

Results and Discussions

The analysis of variance showed a significant interaction effects of PVP and genotypes (p=0.001) for percent of survived and dead explants due to phenolic oxidation. Data of unchanged (neither survived nor dead) explants was not included in the analysis. Among the different concentrations of PVP, the highest percentages; 100% and 80% of survived explants were obtained at PVP concentration of 0.3 gL⁻¹ for C86-12 and 0.2 gL⁻¹ for C86-56 respectively.

As the concentration of PVP increased (Table 1) from 0.0 gL⁻¹ to 0.3 gL⁻¹, the percentage of survived explants of C86-12 significantly increased from 0% to 100% then declined to 40% as the PVP concentration increased from 0.3 gL⁻¹ to 0.5 gL⁻¹. However, the percentage of dead explants was declined from 80% to 0% then increased to 60%. For C86-56, the percentages of survived explants increased from 6.67% to 80% and then dropped to 40% as the concentration of PVP increased from 0.0 gL⁻¹ to 0.2 gL⁻¹ and 0.3 gL⁻¹ to 0.5 gL⁻¹ respectively. Hence, increasing the concentration of PVP beyond 0.2 gL⁻¹ and 0.3 gL⁻¹ is not economical for C86-56 and C86-12 respectively.

The best result of this experiment, i.e., PVP at a concentration of 0.3 gL⁻¹ for C86-12 is in agreement with the result obtained by Shukla RK et al. [15] cultured shoot tips explants on MS media containing 0.5 mgL⁻¹ IBA, 2 mgL⁻¹ BAP+0.5 mgL⁻¹ IAA, and 0.5 mgL⁻¹ BAP+0.5 mgL⁻¹ A₃ with 0.3 gL⁻¹ of PVP. They indicated that though the cultures showed browning after five days of culturing, most of the explants were survived. In this study, the best results obtained for both genotypes are in contrast to the results reported by Huang CM et al. [16] and Michael PS et al. [21]. Huang CM et al. [16] found 60% and 40% browning free explants for two different genotypes at a PVP concentration of (0.5-1) gL⁻¹. This could be due to genotypic differences among the materials used. MS medium supplemented with 3 mgL⁻¹ of 2,4-D+0.5 gL⁻¹ PVP+0.5 mgL⁻¹ BAP+100 mL⁻¹ coconut water+8% agar resulted in successful initiation of large embryogenic callus ranging from 80 to 90% which were free of browning [21]. This difference may be happened due to the difference in genotypes and the type of *in vitro* regeneration path used (Table 2).

The ANOVA revealed that the interactions between concentrations of AC and genotypes were significantly influenced the percentage of survived and dead explants (p=0.001). Data of unchanged (neither dead nor survived) explants was not included in the analysis. The least percentages of survived explants were observed on MS medium without application of activated charcoal for both genotypes. The highest percentages of survived explants were observed on MS medium

Antioxidant (gL ⁻¹)	Genotype			
	C86-12		C86-56	
Levels of PVP	% Survived ± SD	% Dead(B) ± SD	% Survived ± SD	% Dead (B) ± SD
0	0.0 ^f ± 0.00	80.00 ^a ± 0.00	6.67 ^f ± 0.84	86.67 ^a ± 0.84
0.1	20.0 ^e ± 0.00	80.00 ^a ± 0.00	20.00 ^e ± 0.00	80.0 ^a ± 0.00
0.2	40.0 ^d ± 0.017	60.00 ^b ± 0.00	80.00 ^b ± 0.00	0.0 ^e ± 0.00
0.3	100.0 ^a ± 0.00	0.00 ^e ± 0.00	80.00 ^b ± 0.00	20.0 ^d ± 0.00
0.4	60.0 ^c ± 0.00	40.00 ^c ± 0.00	40.00 ^c ± 0.00	40.0 ^c ± 0.00
0.5	40.0 ^d ± 0.58	60.00 ^b ± 0.00	40.00 ^d ± 0.017	40.0 ^c ± 0.58
CV%	7.6	6.81	7.6	6.81

PVP=Poly vinyl pyrrolidone; B=Browning (phenolic oxidation).

*Values for percent of explants survived and dead given as mean ± SD.

*Numbers within the same column with different letter(s) are significantly different from each other according to REGWQ mean separation method at p ≤ 0.05

Table 1: Effects of PVP in preventing browning.

supplemented with 0.4 gL⁻¹ and 0.3 gL⁻¹ of AC for C86-12 and C86-56 respectively. For C86-12, increment of AC concentrations from 0.3 to 0.5 gL⁻¹ showed non-significant effect on percentage of survived explants. However, concentrations of 0.3 gL⁻¹ and 0.4 gL⁻¹ of AC resulted in 60% death of explants. At 0.4 gL⁻¹ of AC only 40% of explants were died due to the effect of phenolic oxidation which is by far better than that of 0.3 gL⁻¹ and 0.5 gL⁻¹ of AC. Even though the percentage of survived explants is the same with 0.3 gL⁻¹ and 0.4 gL⁻¹ of AC for C86-56, the percentage of dead explants is 53.4% at 0.3 gL⁻¹ and 60% at 0.4 gL⁻¹ of AC respectively.

As the concentration of AC increased from 0.0 gL⁻¹ to 0.4 gL⁻¹ for C86-12, percentage of survived explants increased from zero to 46.6% whereas for C86-56 the increment was from zero to 40% as the concentration of AC increased from 0.0 gL⁻¹ to 0.3 gL⁻¹. Increasing the concentrations of AC beyond 0.4 gL⁻¹ for C86-12 and 0.3 gL⁻¹ for C86-56 showed a decreasing trend in percentage of survival of explants. This could be due to fact that AC is not only adsorb plant growth inhibitory substances (phenolics) in medium produced by explants, but also adsorb the plant growth regulators and organic substances that are very important for explants to grow [22].

Results obtained at 0.4 gL⁻¹ for C86-12 and 0.3 gL⁻¹ for C86-56 was similar to the result reported by Huang CM et al. [16]. They reported that AC concentrations from 0.2-0.5 gL⁻¹ resulted in 80% browning free (survived) explants. However, our result contradicts with the result reported by Manchanda P et al. [23] found that MS medium supplemented with 2 gL⁻¹ AC controlled the effect of phenolic oxidation on the medium and furthermore it increased percent of somatic embryogenesis and percent of regeneration from 80.21 to 84.88% and

75.15 to 81.22% respectively. This difference could be happened due to genotypic difference and the type of regeneration path followed.

Conclusion

In line with the current findings, it is possible to deduce that the two genotypes may have different concentrations of phenolics and polyphenol oxidase enzyme; that is why we found different concentrations of PVP and AC for both genotypes to control browning effects on culture establishment stage of micropropagation. Hence, MS medium supplemented with 0.3 g⁻¹ PVP for C86-12 and 0.2 g⁻¹ PVP for C86-56 and 0.4g⁻¹ AC for C86-12 and 0.3 g⁻¹ AC for C86-56 were obtained to be the optimum concentrations to control effects of phenolic oxidation. These concentrations can be used to have maximum survival percentage of plantlets during culture establishment stage of micropropagation to have enough starter culture for multiplication stage (Figure1).

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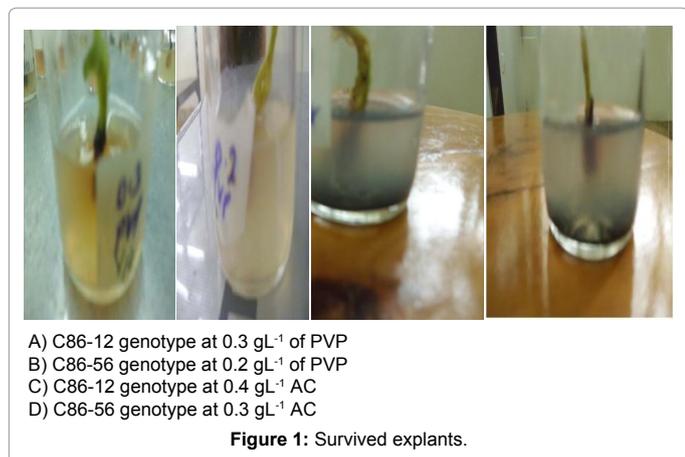
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Antioxidant(gL ⁻¹)	Genotype			
	C86-12		C86-56	
Levels of AC	% Survived ± SD	% Dead (B) ± SD	% Survived ± SD	% Dead(B) ± SD
0.1	20.00 ^b ± 0.00	73.40 ^{bc} ± 0.58	0.00 ^c ± 0.00	86.6 ^{ab} ± 0.58
0.2	20.00 ^b ± 0.00	60.00 ^{cd} ± 0.00	20.00 ^b ± 0.00	80.0 ^b ± 0.00
0.3	40.00 ^a ± 0.00	60.00 ^{cd} ± 0.00	40.00 ^a ± 0.00	53.4 ^{ab} ± 0.58
0.4	46.60 ^a ± 0.58	40.00 ^e ± 0.00	40.00 ^a ± 0.00	60.0 ^{cd} ± 0.00
0.5	40.00 ^a ± 0.00	60.00 ^{cd} ± 0.00	20.00 ^b ± 0.00	60.0 ^{cd} ± 0.00
CV%	7.53	8.51	7.53	5.81

AC=Activated charcoal; B=Browning (phenolic oxidation).

*Values for percent of explants survived and dead given as mean ± SD.

Table 2: Effects of AC in preventing browning.



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