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Free amino acids and proteins dynamics in somatic embryogenesis of African pearwood

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Abstract

African pearwood (*Baillonella toxisperma* Pierre) is one of the biggest trees of the Central Africa rainforest. It offers number of uses but the species is classified as vulnerable. This study is conducted in view of its domestication via somatic embryogenesis. Here we analyzed the variations of free amino acids, soluble and ionically bound proteins during different stages of somatic embryogenesis in half-strength Murashige and Skoog and in half-strength Driver and Kuniyuki media. In both media respectively, the endogenous levels of free amino acids, soluble and ionically bound proteins respectively were low in embryogenic calli [(0.30, 0.72); (0.15, 0.72); (0.09, 0.35) mg/g FM] then increased significantly in globular embryos [(1.60, 0.93); (0.39, 5.35); (0.17, 1.26) mg/g FM)]. Finally, the levels of all these somatic embryogenesis markers decreased significantly in bipolar embryos [(1.41, 0.54); (0.24, 1.89); (0.20, 0.84) mg/g FM] excepted in MS/2, in which ionically bound proteins content increased to 0.20 mg/g FM. Free amino acids, soluble and ionically bound proteins content increased to 0.20 mg/g FM. Free amino acids, soluble and ionically bound proteins content increased to specific types of those biochemical markers.

Keywords: Baillonella toxisperma, somatic embryos, amino acids, proteins.

INTRODUCTION

African pearwood, *Baillonella toxisperma* Pierre (Sapotaceae), is a species found in the Central Africa rainforest. It is among the biggest trees of the continent and is distributed from southern of Nigeria to the Democratic Republic of Congo (Vivien et al., 1985). The plant is sought by the forest operators for the quality of its wood as well as by the local populations for its fruits and seeds, from which quality oil can be extracted. Indeed, *B. toxisperma* wood exploitation is estimated at a rate of 100 000 m³ per year (ATIBT, 2006). Data concerning fruit harvestings remain much rarer (Vermeulen et al., 2005).

Abbreviations

MS/2 (Half-strength Murashige and Skoog medium); **DKW/2** (Half-strength Driver and Kuniyuki medium; **EC** (Embryogenic calli); **GSE** (Globular Somatic Embryos); **BSE** (Bipolar Somatic Embryos); **FM** (Fresh Weight Matter).

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This double lust could cause a depletion of the species in a medium-term (Debroux, 1998). The species is classified as vulnerable and threatened of extinction in its ecological systems (Newton et al., 2003). Its domestication is therefore essential and an adequate method of plantlets multiplication is necessary.

Plants regeneration via somatic embryogenesis offers a particular advantage which consists to yield new plants with more stable genome (Roja Rani et al., 2005). This technique is an alternative pathway for the propagation of African pearwood (Sanonne et al., 2012). Somatic embryo development reposed on biochemical and physiological principles that are essential to be understood (Silveira et al., 2008).

Amino acids are the principal providers of organic nitrogens incorporated by all plants during their metabolism. The endogenous contents of several important amino acids increased throughout somatic embryogenesis process (Sen et al., 2002) however it was observed a rise in globular and torpedo stages and a fall in germinating embryos (Joy et al., 1996). Some



Figure 1. Major stages of somatic embryogenesis in *Baillonella toxisperma* indicated by arrows: a=Embryogenic calli, b=Globular somatic embryos, C=Bipolar somatic embryos, bar=1cm,

exogenous amino acids were used as *in vitro* supplements (Niemenak et al., 2008).

The contents of soluble proteins enhanced gradually during embryo development and attain their maximum levels at the last mature stage (Silveira et al., 2004). The accumulation of proteins was underlined as a

biochemical marker of efficient somatic embryo development (Griga et al., 2007). The bound proteins are mainly known to have structural roles in this developmental process (Showalter, 2001).

The aim of this study is to analyze the role of free amino acids, soluble and ionically bound proteins at different stages of somatic embryogenesis in African pearwood.

MATERIAL AND METHODS

Plant material

The contents of amino acids, soluble and bound proteins were measured in Embryogenic calli (EC), Globular Somatic Embryos (GSE) and Bipolar Somatic Embryos (BSE). They were obtained indirectly from leaf explants of about 2 weeks old after bud opening of plantlets grown from germinating seeds of *Baillonella toxisperma*. Disinfection and seeding were done using Sanonne et al. (2012) method.

Culture media preparation and obtainment of different stages of cultures

The culture media used were: (1) half strength solid Murashige and Skoog (1962) mineral salts (MS/2) containing 4.5% sucrose, 0.6% agar and 1 ml/l Morel and Wetmore (1951) vitamins; and (2) half strength solid Driver and Kuniyuki (1984) mineral salts (DKW/2) containing 250 mg/l glutamine, 100 mg/l myoinositol, 20 g/l glucose, 25 μ g/l TDZ, 2 g/l phytagel and 1 ml/l of DKW vitamin solution.

Embryogenic Calli (Figure 1a) were inducted by culturing leaf explants for 28 days in MS/2 or DKW/2 supplemented with 0.5 media mg/l of 2.4dihlorophenoxiacetic acid (2.4-D) and 0.5 ma/l benzylaminopurine (BAP). To induce the formation of Globular Somatic Embryos (Figure 1b), calli were transferred for 60 days in the same enriched MS/2 or DKW/2 basal media containing 2 or 3 mg/l of 2.4-D. The Globular Somatic Embryos were subcultured over 97 days in the same media containing 0.5 or 1 mg/l 2.4-D supplemented with 0.5 mg/l abscisic acid (ABA) for differentiation and maturation of Bipolar Somatic Embryos (Figure 1c).

All culture media with pH adjusted to 5.6 were sterilized by autoclaving at $115 \,^{\circ}C/30$ min. The culture room temperature was $25 \pm 2 \,^{\circ}C$ under 40 μ mol/m²/s white fluorescent light and 16 h lighting photoperiod.

Extraction and analysis of amino acids

The fresh material (1g) constituted of EC, GSE or BSE was ground in 5 ml of ethanol 80° . Amino acids were then extracted using reflux technique in boiling ethanol for 30 min. After decanting, the supernatant was filtered with Wattman paper n°3. The filtrate was collected and the residual was used to repeat the extraction. The two



Figure 2. Variations of free amino acids contents following the different stages of somatic embryogenesis in MS/2. The same letters indicate no significant difference (LSD multiple range test, p = 0.05). EC (Embryogenic calli), GSE (Globular Somatic Embryos), BSE (Bipolar Somatic Embryos).

mixed filtrates constituted the raw extract of amino acids that were measured using ninhydrin method (Yemm and Cooking, 1955). The absorbance of purplish bruise complex was read at 570 nm. The standard curve was established using 0.1 mg/ml of glycine.

Extraction and analysis of soluble and bound proteins

Fresh material (1g) like previously was ground in 2 ml cold Tris-maleate buffer 0.05M, pH 7 containing mannitol 0.5M. The homogenate was incubated at 4 °C for 20 min and centrifuged at 6000 g_n for 40 min. The supernatant was collected and constituted the soluble fraction of proteins. The residual was retaken two times in the previous buffer for 20 min with at each time centrifugation at 6000 g_n for 20 min and elimination of the supernatant. The residual was mixed and incubated in 1 ml cold Trismaleate buffer 0.01M, pH 7 containing sodium chloride 1M at 4 °C during 40 min then centrifuged at 6000 g_n for 40 min. The supernatant was collected and constituted the ionically bound fraction of proteins. The quantity of proteins was determined according to Bradford (1976). The absorbance of the blue complex was read at 595 nm against the white. The standard curve was obtained using bovine albumin serum 1 mg/ml.

Data analysis

After statistically significant difference between average contents of biochemical markers globally obtained using ANOVA ($P \le 0.05$), LSD multiple range tests (P=0.05) was

used to compare these means to each other. The analyses were performed using "Statgraphics plus" software (5.0 version).

RESULTS

Changes in amino acids content

In MS/2 medium, the low amino acids levels in embryogenic calli (0.30 mg/g FM) increased significantly (p = 0.05) in globular and bipolar somatic embryos with average levels of 1.60 and 1.41 mg/g FM respectively (Figure 2).

Amino acids contents in medium DKW/2 also showed significant variations between the different stages of cultures. In embryogenic calli, amino acid content was twice that observed on MS/2 medium i.e. 0.72 mg/g FM. In globular somatic embryos, a significant increase was observed in amino acid levels compared to the previous step with an average value of 0.93 mg/g FM. At bipolar somatic embryos stage, there was a significant decrease in amino acid levels (0.54 mg/g FM) compared to the quantities obtained previously (Figure 3).

Changes in soluble proteins content

In MS/2, the contents of soluble proteins varied significantly (p = 0.05) following development stages. The minimal quantity (0.15 mg/g FM) was obtained in embryogenic calli. From this initial stage, soluble proteins amounts increased significantly (p = 0.05) in the two next



Figure 3. Variations of the contents of free amino acids following the different stages of somatic embryogenesis in DKW/2. The same letters indicate no significant difference (LSD multiple range test, p = 0.05). EC (Embryogenic calli), GSE (Globular Somatic Embryos), BSE (Bipolar Somatic Embryos).



Figure 4. Variations of soluble proteins contents following the different stages of somatic embryogenesis in MS/2. The same letters indicate no significant difference (LSD multiple range test, p = 0.05). EC (Embryogenic calli), GSE (Globular Somatic Embryos), BSE (Bipolar Somatic Embryos).

steps with means of 0.39 mg/g FM and 0.24 mg/g FM respectively in globular and bipolar somatic embryos stages (Figure 4).

At each stage of development, the amounts of soluble proteins were higher in DKW/2 than MS/2. However, a similar behavior was observed that is a low level (0.72 mg/g FM) in embryogenic calli step, an increase in globular embryos stage (5.35 mg/g FM) and a decrease in bipolar embryos stage (1.89 mg/g FM) (Figure 5).

Changes in bound proteins content

In MS/2, the ionically bound proteins to walls and membranes varied in different steps of somatic embryogenesis. In embryogenic calli step, ionically bound protein content was 0.09 mg/g FM. In globular embryo stage, its amounts increased significantly compared to the previous step to 0.17 mg/g FM. In bipolar embryos, the highest ionically bound protein content of 0.20 mg/g FM has been recorded (Figure 6).



Figure 5. Variations of soluble proteins contents following the different stages of somatic embryogenesis in DKW/2. The same letters indicate no significant difference (LSD multiple range test, p = 0.05). EC (Embryogenic calli), GSE (Globular Somatic Embryos), BSE (Bipolar Somatic Embryos).



Figure 6. Variations of the contents of ionically bound proteins following the different stages of somatic embryogenesis in MS/2. The same letters indicate no significant difference (LSD multiple range test, p = 0.05). EC (Embryogenic calli), GSE (Globular Somatic Embryos), BSE (Bipolar Somatic Embryos).

Initially in embryogenic calli, ionically bound proteins content was low in DKW/2 (0.35 mg/g FM). It increased significantly in the next two steps. In globular embryos, there was an accumulation with a mean content of 1.26 mg/g FM. In bipolar embryos stage, bound proteins levels decreased significantly compared to the previous stage at 0.84 mg/g FM (Figure 7).

DISCUSSION

Endogenous levels of amino acids were evaluated in this study. It was reported that they play a key role in embryos development (Merkle et al., 1995). Among the factors that modulate biochemical and physiological processes of somatic and zygotic embryogenesis, amino



Figure 7. Variations of the contents of ionically bound proteins following the different stages of somatic embryogenesis in DKW/2. The same letters indicate no significant difference (LSD multiple range test, p = 0.05). EC (Embryogenic calli), GSE (Globular Somatic Embryos), BSE (Bipolar Somatic Embryos).

acids represent the first step in nitrogen assimilation (Ortiz-Lopez et al., 2000). In MS/2 medium, free amino acids contents was low in embryogenic calli and then increased significantly during globular and bipolar embryos formation. These results are similar to those obtained in Pinus patula (Malabadi and van Staden, 2005) and in Theobroma cacao (Niemenak et al., 2008). In the last two stages of embryo development, there was no significant difference between the levels of amino acids. In DKW/2 medium, the same behavior in terms of changes in amino acid levels for the first two stages was observed, that is lower in embryogenic calli than in globular embryos. At bipolar embryos stage, there was a significant drop of amino acids level. A decrease in the levels of amino acids from globular embryos stage was reported in Acca sellowiana (Booz et al., 2009). There was in this case, an extensive mobilization of amino acids in the synthesis of storage proteins (Santa-Catarina et al., 2006). However, some specific analyzes should be done to determine the roles of specific amino acids. In fact, studies have shown that certain amino acids may be more efficient than others in this process (Garin et al., 2000; Booz et al., 2009).

There are several studies on the biosynthesis and accumulation of soluble proteins during embryogenesis process. In *Baillonella toxisperma*, the amounts of soluble proteins were low in embryogenic calli stage and increased significantly in globular and bipolar embryos stages in MS/2 as well as in DKW/2 media. The similar variation was reported in *Pisum sativum* (Griga et al., 2007). However, in both types of media, the soluble protein contents dropped in bipolar embryos stages

compared to their contents in globular embryos. That observation was the opposite reverse of that of Silveira et al. (2008) and Cangahuala-Inocente et al. (2009) who noted a gradual increase in the levels of soluble proteins from globular to cotyledonary embryos stages in Araucaria angustifolia and Acca sellowiana respectively. Indeed, it has been found that the process of histological differentiation of embryos is closely associated with changes in proteins, carbohydrates and lipids synthesis and mobilization (Griga et al., 2007; Cangahuala-Inocente et al., 2009). In general, a progressive accumulation of proteins is observed during embryo development (Sallandrouze et al., 2002). These substances whose levels vary during different stages of development of cell cultures are involved in some transduction signals or are used as substrates or regulators of growth and morphogenesis (Lulsdorf, 1992; Jimenez, 2001).

The variations of ionically bound proteins were studied. In embryogenic calli steps their quantities were low. The bound proteins as ionic or covalent to walls and membranes which include **HRGPs** mainly (hydroxyproline-rich glycoproteins) or extensins, AGPs (arabinogalactan proteins), GRPs (glycine-rich proteins) and PRPs (proline-rich proteins) play a structural role (Cassab, 1998). Therefore, we can assume that cells were still young and the formation of their walls and membranes were not yet complete. Cassab and Varner (1998) reported that in the early stages of development of zygotic embryos, it is impossible to detect extensin whose role is to promote extensibility of walls while it is highly concentrated in embryos of mature seeds. lonically

bound proteins levels increased significantly in globular and bipolar stages, which may be associated with progressive maturation of embryos.

This study revealed that in MS/2 as well as in DKW/2 media, endogenous levels of free amino acids, soluble and ionically bound proteins were low in embryogenic calli then increased significantly in globular embryos. The levels of all these somatic embryogenesis markers decreased significantly in bipolar embryos excepted in MS/2, in which ionically bound proteins content remained high.

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