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Isolation and culture of protoplasts of Côte d'Ivoire's pearl millet (*Pennisetum glaucum* (L) R) varieties

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ABSTRACT

Objective: Protoplasts are the ideal material for genetic transformation of plants. This requires that the protoplasts have the ability to regenerate whole plants. The objective of this study is to isolate protoplasts from cell suspensions and test their ability to regenerate embryogenic calli and plants.

Methodology and results: Protoplasts were isolated with different enzyme combinations, from cell suspensions of millet, *Pennisetum glaucum*. Obtaining callus from protoplasts was carried out on various media at various pH with different glucose concentrations. Approximately 13,106 protoplasts / g were isolated from cell suspensions. Calli were regenerated by culturing the protoplasts at pH 5.8, at a concentration of 0.7 M glucose in either the liquid medium or solid medium. The plating efficiency of protoplast is from 0.012 to 0.013 in solid medium containing 0.6% agarose. No plant has been regenerated from calli provided from protoplasts. All plant regeneration attempts resulted in the formation of globular structures. Cytological studies have shown that the calli derived from protoplasts are formed with 50% of multinucleate cells.

Conclusion and application of results: This study allowed isolating protoplasts, regenerating embryogenic calli from protoplasts of millet varieties of Côte d'Ivoire and highlighting one of the causes of the recalcitrance of the grass crop culture regeneration from provided protoplasts. This study will allow genetic transformation of millet varieties by using protoplasts.

INTRODUCTION

Protoplast culture is a key element in genetic engineering development such as somatic hybridization and gene transfer. However, the application of these techniques is possible if reproducible methods for plants regeneration from protoplasts are available. In cereals, the first calli from protoplasts were obtained with rice (Deka *et al*., 1976), maize (Chourey *et al*., 1981), sorghum (Chourey *et al*., 1985) and wheat (Yasuyuki, *et al*., 1988). Plant regeneration from protoplasts was established in *Oriza sativa* L. (Datta *et al*., 1990; Baset *et al*., 1993); in *Zea maize* (Petersen *et al*., 1992); in *Triticuma estivum* L. (Vasil *et al*., 1990, Chang *et al*., 1991); in *Hordeum vulgare* (Ziauddin, 1992); in *Sorghum vulgare* (Zhi-Ming *et al*., 1990); in *Saccharum officinarum* L. (Srinivasan, 1986). Although the majority of these works have used cell suspension as the main source of protoplasts,

several other approaches have also been used to regenerate plants. Indeed, protoplasts have been regenerated from rice mesophyll (Gupta and Pattanayak, 1993) and from Tylophoraindica mesophyll (Thomas, 2009). Within the genus Pennisetum, Vasil *et al*. (1979) obtained calli from cell suspension protoplasts of *P. americanum*, and they regenerated plantlets. Calli and plantlets were also obtained respectively with protoplasts of *P. squamulatum* (Gupta *et al*., 1988) and protoplasts of *P. purpureum* Schum (Vasil *et al*., 1983). The conditions for protoplasts isolation of mésophyles of *P. glaucum* and *P. purpureum* were reported by Timbo De Oliveira *et al*. (2010). Unlike other grasses or cereals such as rice, where the experiments were reproducible, work on Pennisetum has not yet led to reproducible regeneration from protoplasts (Mtili, 1990). Except for the use of isolated protoplasts for the expression of foreign genes (Tiécoura *et al*., 2001), studies on protoplasts of pearl millet, *Pennisetum glaucum*, varieties of Côte d'Ivoire are scarce. In this work, we present protoplast culture assays of pearl millet, *Pennisetum glaucum*, varieties of Côte d'Ivoire, the conditions of isolation, culture and plant regeneration from protoplasts.

MATERIEL AND METHODS

Vegetable material: Protoplasts used, were derived from embryogenic cell suspensions obtained from embryogenic calli of NE (northeast) millet variety (Tiécoura *et al*., 2014a).

Protoplast isolation: Isolation of protoplasts was performed using the methods of Potrykus (1977) and Shillito (1983) with some modifications: After centrifugation of cell suspension at 160xg / 2-3min, the pellet was weighed to determine the mass of fresh material. The pellet was suspended in protoplast culture medium containing different enzymes: Cellulase "Onozuka" RS (1-4%), R-10 macerozyme (Yakult Honsha Co. Tokyo) (0.5 to 1%), pectolyase Y -23 (SeishinParmaceutical Co. Tokyo) (0.2 to 0.5%), MES (Boehringer) (0.5%), mannitol and CaCl2 (0.25M). The medium at pH 5.6 is sterilized by a filtration on 0.2µm filter. The mixture was digested under stirring (40-50rt / min) in the dark for at least four hours at 25 ° C. After digestion of the skeletal wall, two volumes of a CaCl2 solution (0.25M), MES (0.5%) were added and the whole mixture filtered successively on sieves of 630, 250 and 25 µm. The filtrate was centrifuged for 5min at 160xg and the pellet was recovered in the base solution that was used in the enzyme solution with mannitol (0.6M). Protoplasts were washed 2-3 times with the same solution and taken up in 25ml before being counted. Viable protoplasts, viability according to Larking (1976), were cultured.

Protoplast culture: Two media were used: liquid medium (Coulibaly *et al.,* 1986, Xia *et al*., 1992) and solid medium (Shillito, 1983, Petersen *et al*., 1992).

- **Culture in liquid medium:** The protoplasts were plated at a concentration of 10^5 to 10^6 / ml in MS medium or N6 containing different glucose concentrations (0,6M; 0,7M) and at various pH (5.2 to 6). The culture is maintained in the dark at 25 ° C. After 7 to 10 days, the glucose concentration is halved with fresh medium without sugar, or culture is centrifuged and the pellet is taken up in fresh medium with glucose reduced by half. After one week, the sugar concentration was reduced to the concentration of the culture medium of the cell suspension (0.1 M) to allow microcalli to develop. The microcalli that appear were transferred on solid media MS or N6.
- **Culture in solid medium (Sea plate, FMC BioProduction, USA):** The washed protoplasts were taken up by the culture medium (twice in concentration) at a concentration of 10^6 / 500µl. To one volume of protoplasts was added a volume of agarose (twice in concentration). The gently homogenized mixture was placed in the Petri dish forming an agarose "pancake" that is grown with or without feeder cells.
- **Culture without feeder cells (Potrykus, 1977, Shillito, 1983):** The "pancake" was grown without culture medium or immersed in the culture medium and cultivated on a rotating table at 45 rpm / min. After 7 to 10 days, the cakes, dry or not, were immersed in the culture medium with reduced sugar concentration in half. At the second week, the "pancake" was immersed in the medium of normal cell culture. One or two weeks later, the "pancake" was placed on the solid medium. Developing microcalli was transferred on the solid medium.
- **Culture with feeder cells: the "nursing" method (Petersen *et al*., 1992, Kamo *et al*., 1987):** The "pancake" was immersed in a culture medium containing feeder cells (1 volume of growth medium, double concentration of sugar and 1 volume of cell suspension) and cultivated on rotating table. After a week, fresh medium with reduced sugar concentration in half was

used. In the case of the submerged culture, 80% of feeder cells were removed. After one or two weeks of culture, the "pancake" was washed several times with distilled water and then transferred to solid medium MS or N6.

Plants regeneration: For regeneration, the MS medium (0.5 / 2) was used (Tiécoura *et al*., 2014b). Calli derived from protoplasts grown in liquid or solid media were used to regenerate plants. Before transferring to the regeneration medium, the calli were cultured for 1-8 weeks on different maintenance media (Table 3).

Calli cytogenetic study: DAPI test (Coleman *et al.*, 1985): The test uses two fluorochromes, mithramycin and 4, 6-Diamidino-2-phenylindole. They bind to DNA and used to locate the core in the dividing cells.

RESULTS

Enzyme combination protoplast isolation: Three days after subculture, the cell suspension was digested with one of three combinations of enzymes for 1 to 6 hours. Table 1 shows the rates of cells having a complete digestion for a given protoplast, with their embryogenic characters, as observed on figure 4A. After one hour of digestion, all the protoplasts isolated (100%) still exhibit wall pieces. For the enzyme combination (1, 0.5, 0.2), about 6 hours are required to have about 80% protoplasts. For the enzyme combinations (2, 0.5, 0.2) and (4, 1, 0.5), four hours of digestion were required to have about 90% protoplasts. The second enzyme combination (2, 0.5 and 0.2) is used for the further isolation of protoplasts after digestion of at least 4 hours.

Table 1: Evolution of completely digested cell rate (%) according to the enzyme combination and the incubation time

Enzyme combination			Time of incubation (hours)					
Ce %	**Ma %**	**Pe %**	**1**	**2**	**3**	**4**	**5**	**6**
1	0.5	0.2	0	15	30	50	70	80
2	0.5	0.2	0	40	70	90	90	90
4	1	0.5	0	40	80	90	90	90

Ce = cellulase; Ma = macerozyme; Pe = pectoliase

Age of subculture and protoplasts isolation: The subcultures of 1 to 6 days were used to isolate the protoplasts. Figure 1 shows the yields of protoplasts expressed as $X10^6$ per gram of fresh weight ($X\ 10^6 \times g^{-1}$ FW). Protoplasts yield varies with subculture times. At the first day, protoplasts yield is of 7.10^6/g FW. It increases and reaches a peak at the third day (14.10^6/g FW) and then decrease to reach its lowest value ($0.07.10^6$/g FW) at the 6th day.

Viability of protoplasts: Xia culture media at 0.6M or 0.7M glucose were prepared at different pH. The viability rate and the rate of protoplasts with embryogenic characters were estimated after a week of culture. The results are in Table 2.

Table 2: viability of the protoplasts according to the pH and the glucose concentration

		pH				
[glucose]	Protoplasts	5.2	5.4	5.6	5.8	6
0.6M	viability	±	++	++	++	++
	E C	-	-	±	±	±
0.7M	viability	±	++	++	++	++
	E C	-	±	++	++	++

EC = embryogenic characters; - = 0%; ± = 50%; ++ = >80%

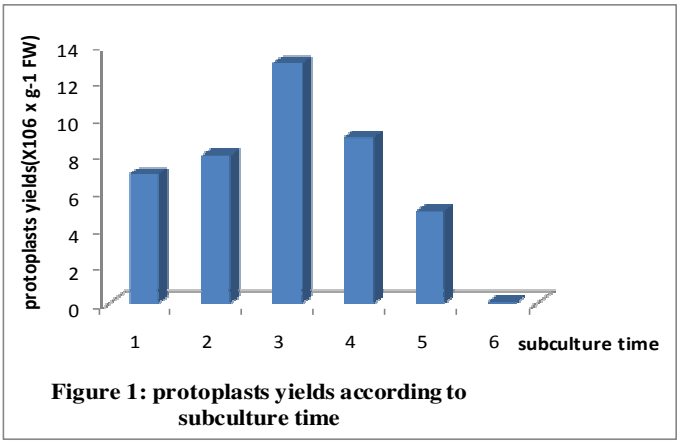

Figure 1: protoplasts yields according to subculture time

The viability of protoplasts was observed as shown on the figure 4 B. At pH5.2, the viability of protoplasts is 50% whatever the concentration of glucose. For the other pH, it is >80% whatever the concentration of glucose. For embryogenic character of protoplasts at glucose 0.6M, protoplasts are not viable (0% of viability) with pH5.2 and pH5.4. For the other PH, 50% of protoplasts are viable at glucose 0.6M. With glucose 0.7M, 0% of protoplasts retain their embryogenic character at pH5.2. This rate is 50% at pH5.4 to over 80% for pH5.6.

Kinetics of the first cell divisions: Fractions 1.10^6 protoplasts are cultured in the medium supplemented Xia 2.4-D (2mg / l). The medium is either liquid or solid (agaros 0.6%). The rate of cell divisions based on the culture time is presented in Figure 2. The first divisions of protoplasts are observed until the fourth day (5-15%) (Figure 4C). From 10 to 30% after the first week of culture in liquid or solid respectively, the number of divisions increases rapidly to reach 70 to 80% in the second week of culture in liquid or solid respectively. However, almost 20 to 30% of the protoplasts cultured in solid or liquid medium respectively do not divided. In the liquid medium, 5-10% of these protoplasts merged and failed. The fusion process is reduced in the solid culture.

Calli from protoplasts culture: Fractions 1.10^6 protoplasts are cultured in the medium supplemented Xia 2.4-D (2mg / l). The medium is solid with agarose 0.6% or agarose 1.2%. Average of calli developed by culture according to agarose concentration is shown in Figure 3. On media containing 0.6% agarose, MS gives approximately 120 calls per culture and N6 140 callies by culture of 1.10^6 protoplasts. With 1.2% agarose, the average observed was 40 and 50 calli respectively on MS and N6. The plating efficiency varies from 0.012 to 0.014 protoplasts for 0.6% of agarose and 0.004-0.005 protoplasts for 1.2% agarose.

Plant regeneration from calli derived from protoplasts: Cultivated calli (1 to 8 weeks) are transferred to the MS regeneration medium (0.5, 2). The morphology and the ability of calli to regenerate plants are shown in Table 3.

Table 3: Plant regeneration tests with calli derived from protoplasts

N°	Maintenance media	Calli morphology	Regeneration
1	MS(1-1-2.5)	-	- -
2	N6(1-100-25)	-	- -
3	MS(5)	-	- -
4	MS(2.5)+$AgNO_3$(58.9µM)	+	- -
5	MS(0)+ABA(0.2)	+	- -
6	MS(1-1-2.5)+$AgNO_3$	+	- -
7	(1-0-0)+$AgNO_3$	+	- -
8	N6(0)+ABA(0.2)	+	- -
9	X+ coal (0.5%)	+ +	- -

X = any culture medium mentioned above / - = wet and less globular cal / + = less humid and globular cal / ++ = dry and globular cal / -- = no regenerated plants

Nine culture media were used to strengthen the callies before regeneration. The media 1, 2 and 3 gave wet calli regardless globular and non-embryogenic. The media 4 to 8, respectively characterized by the presence of silver nitrate AgNO3 and abscisic acid (ABA) present relatively globular and less wet calli and do not produce plants. The action of coal, leads to globular and dry embryoids (figure 4H) but do not either give plants. Binocular observations show not-compact and snowy globular embryoids where hairs could be observed. Cytogenetic studies of the calli have shown that several cells are without core and other more cells are with several cores (figure 4I-J).

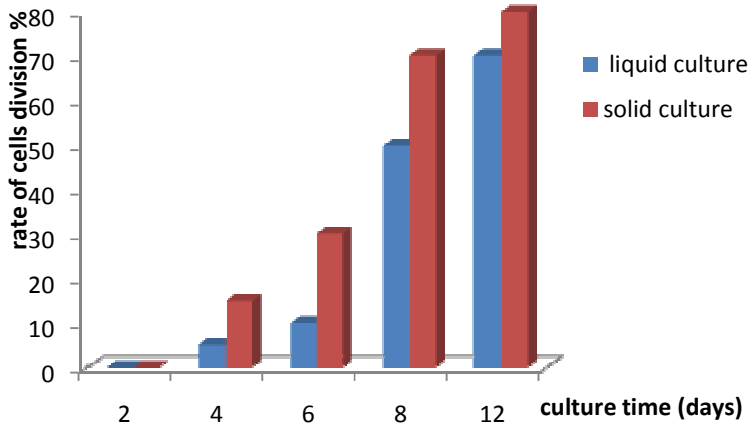

Figure 2: Evolution of cells division according to time and type of culture

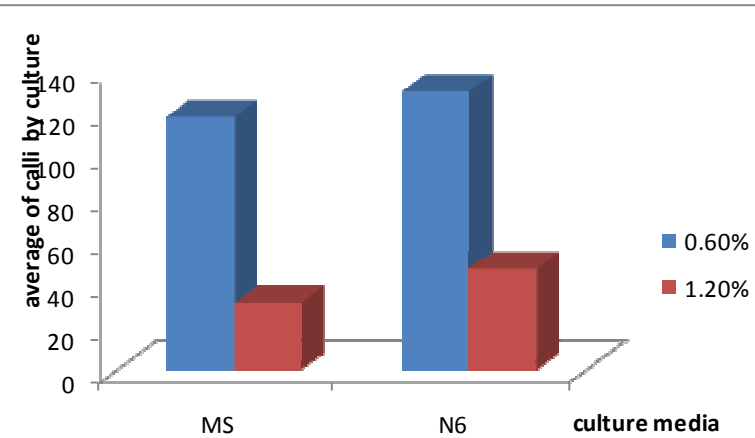

Figure 3: Calli development according to media and agarose concentration

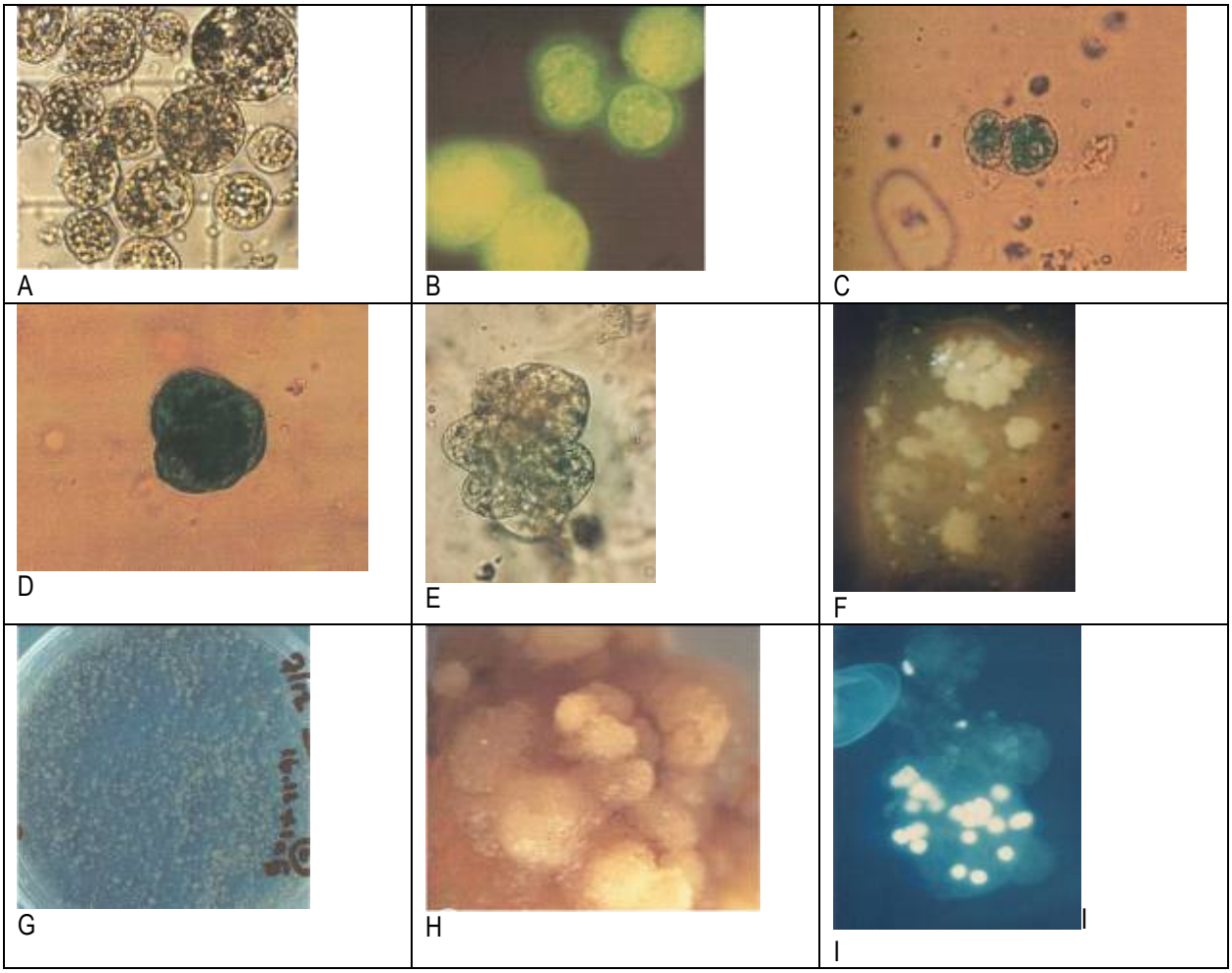

Figure 4: Isolation and protoplasts culture: A= freshly isolated Protoplasts showing dense cytoplasm; B = Viable protoplasts with fluorescein (FDA) staining; C= first division showing wall formation; D-E = successive division gives rise to microcalli; F= calli developed in agarose plating; G = calli developed in liquid plating; H= embryoids structures developed on regeneration medium; I= multineated cells with DAPI staining

DISCUSSION

This study data indicates that enzyme combination that is effective is 2% (w / v) cellulase, 0.5% macerozyme and 0.2 % (w/v) pectoliase. With *Musa spp*, Assani *et al*. (2001) used the combination of 2% (w / v) cellulase, 0.5% Macerozyme, 0.25% (w / v) hemicellulase and 0.25% (w / v) Pectolyase. However, with *Musa spp*, Cavendish Sub-group AAA, Assani *et al*. (2005) used an enzyme combination composed of 1.5% (w / v) cellulase and 0.15% (w / v) pectolyase. Enzymes are substances that can digest cellulose and pectic substances of the cell wall and release the protoplasts. The composition in cellulose and pectic substances of cells are species dependant (Wertz, 2011). This explains the differences in enzyme combinations. The first protoplasts were released after 4 hours of incubation. Although this time is usually not determined, it is nevertheless important for protoplasts culture. Thus, for *P. glaucum*, cells were generally left for 12 hours in the enzyme solution (Xia *et al*., 1992). For Banana, 15 to 17h of digestion or 12 to 14 hours of digestion were requested (Assani *et al.,* 2001; 2005). For Kappaphycus, 4 hours of digestion were requested with 20% abalone enzyme and 12% of cellulose (Zhang Si *et al*., 2014). Enzyme combinations are also a function of the pectocellulose composition in the walls. This diversity in the enzyme composition reflects the complexity of cell wall composition (Wertz, 2011). Hence, it is necessary to reduce the digestion time to avoid the possible toxicity and effect of the enzyme on protoplasts viability (Raoudha, 2001). An accumulation of these substances in the protoplast may affect survival. The variation of the

average value of the protoplast yield, depending on the time of subculture, is due to the quality of the wall and the amount of cells. In the early days of the subculture, the division rate was exponential. From the third day, the divisions diminished given the saturation and the impoverishment of culture medium (Tiécoura *et al*., 2014a). In these first days of divisions, the cells were young with a primary wall called growth wall (Hongo *et al*., 2012). The primary wall is simple in its constitution and easy to be digested by enzymes. The amount of young cells increases, there is also an increased yield of protoplasts. In the period of saturation of the culture medium, cells are composed of secondary or tertiary wall (Kim *et al*., 2012; 2014). This wall with inlays substances is a secondary cell wall that is tough and more difficult to digest by enzymes and lead to the fall of the protoplast yield despite of the long incubation time. The average value of protoplast performance observed in this study is 2-26 times higher than those reported for other grasses: $0,5.10^6$ x g^{-1} In *Sorghum vulgare* (Zhi-ming *et al*., 1990); $1,4.10^6$ x g^{-1} in *Pennisetum glaucum* (Mtili, 1990); $6 .10^6$ xg^{-1} in *Oryza sativa* (Lee *et al*., 1989) and 0.65.106 g-1in Kappaphycus (Zhang Si *et al*., 2014). The highest value was observed in *Triticum aestivum* with 0.28 to $5 .10^7$x mg^{-1} fresh weight (He *et al*., 1992). This variability in the yield of protoplasts confirms the species dependence of cell walls diversity. For the viability of protoplasts, 0.7M concentration of the glucose and a pH of 5.6 seem to be the best for protoplasts viability and embryogenic character conservation. Our parameters determined are similar to those observed in most of the grasses (Mtili 1990, ZhangSi *et al*., 2014). With *Oryza granulata*, the rate of protoplast division is about 25% after one week of culture (Baset *et al*., 1993). Wang *et al*. (1989) found 5-10% among Japonica varieties of male sterile (CMS) Cell division testifies the vitality of protoplasts and indicates that protoplasts recovered their cell wall. Calcofluor colouration reveals that protoplasts cell wall was restored after three to four days. The protoplasts were progressively obtained, their wall, the first sign of cell totipotency. Approximately 20 to 30% protoplasts witch degenerate would be unable to rebuild their cell wall. The division was done either by budding (Xia *et al*., 1992) or by segmentation of the cytoplasm (Vasil *et al*., 1990), or by normal bipartition (Ishak, 1992). In liquid culture, the balance is budding and cytoplasmic partitioning. Segmentation and bipartition were most often observed in solid culture. In both solid and liquid medium, calli were obtained from the protoplasts culture. The production of protoplasts on solid cultures reported in this study presents a great variability in the number of calli obtained. This was the case with the protoplasts cultures of mesophyll *Oryza sativa* (Gupta *et al*., 1993) where 140-250 callies were obtained per culture. For *Saccharum officinarum,* 20 to 72 callies have been reported (Taylor *et al*., 1992). The concentration of agarose seemed to influence the efficacy of the spreading protoplasts. A concentration of 0.6% appear to be more appropriate than 1.2%, since the concentration 1.2% seems to reduce the diffusion of the culture medium nutriments or the metabolites arising from the feeder cells; these metabolites promote calli protoplasts. Nevertheless, the plating efficiency of protoplast values obtained in the present study remain low (0.012 to 0.013) compared to these obtained with other species: for *Zea mays*, with 0.6 or 1.2% agarose, the efficiency is 0.25 (Chourey *et al*., 1981) and 0.8 with 0.4% agarose (Kamo *et al*., 1987). For Triticum aestivum L., with 1.2% agarose, the efficiency is 0.02 to 0.11 (Kasem *et al*., 1993). With 2.5% agarose, the plating efficiency of protoplasts is estimated from 1 to 9.8 with Oryza sativa (Kyozuka *et al*., 1987). Here, it is established that the agarose effect depends on the species. Plants were no regenerated from calli protoplasts. Despite the action of $AgNO_3$, ABA and activated coal leading to drier callies and observation of hair on the surface of callies, true embryos could not be regenerated; hence the absence of plants. The main action of Abscisic acid was to allow proper maturation of embryos germinating. In our case, there were no true embryos; only snow globular embryoids structures appeared. Factors that may explain the non-regeneration of plants from calli protoplasts are numerous. From the cell suspension to obtained protoplasts calli, it takes at least three months. It is established that after three months, plant regeneration rate from liquid culture, drops to 10% (Tiécoura 2014b). This time could not be reduced and could explain why the calli was unable to develop real embryos despite the use of different media. This recalcitrance of graminea was previously reported with *Pennisetum americanum* (Mtili, 1990) and with *Sorghum bicolor* (L) (Ishak, 1992) despite previous reports of the regeneration of protoplasts from these cereals (Vasil *et al*., 1980; Zhi-Ming *et al*., 1990). Regenerative capacity can also depend on the species, varieties and genotypes (Vasil *et al*., 1980). We believe that graminea cell totipotency (see differentiation and dedifferentiation of Poaceae plant cell) is challenging. In the case of *Pennisetum glaucum,* a mitotic disorder can affect totipotency. The possible multi nucleated cells observed may suggest that the calli from protoplasts is a chimeric cell. The chimeric cell behaviour would lead to a possible carcinogenic response of calli, leading to the lost of their

ability to differentiate into regenerative plants embryos. Finally, genes methylation in cells culture may be a factor promoting uncontrolled proliferation blocking cell differentiation in graminea.

CONCLUSION

Protoplasts of millet varieties of Côte d'Ivoire can be isolated, cultured and regenerated in calli. In spite of this multitude of culture media of calli, no calli transferred on medium of regeneration can give plants. The non-regenerating of plants reveals that further studies need to be conducted to better understand the totipotency, to provide solutions to the recalcitrance of graminea relative to plant regeneration from protoplasts. Understanding of this totipotency could provide less expensive ways to improve graminea in general and in particular the Ivorian millet.

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