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Androgenic potential and anther *in vitro* culture of *Lagenaria siceraria* (Molina) Standl an edible-seed cucurbit

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ABSTRACT

Lagenaria siceraria Molina has a high productive potential of fruits but the seeds weight remain low. The aim of this study is to regenerate doubled haploids from androgenesis in order to create genotypes with good agronomic characteristics. For this purpose, flower buds of six accessions namely NI185, NI202, NI215, NI276, NI356 and NI271 were pre-treated at 4 °C for 0, 1, 2, 4, 6, 7 days. To assess the ability of these accessions to induce callus, the anthers excised from the collected floral buds were cultured following two standard protocols referred to as Metwally et al. and Kumar and Murthy, respectively. Histological sections were performed with buds of different sizes in order to select pollen at uni-nucleate stage, the most appropriate for the androgenesis. The histological sections showed that the pollen is at uni-nucleate stage when the bud size varied from 5 to 7 mm. No callus production was observed with the protocol of Metwally et al. whereas a significant formation of callus were expressed on medium corresponding to the protocol of Kumar and Murthy. The best rate (79%) of callus induction was obtained after one and two days of pre-treatment at 4 °C. The highest rate of callus induction was observed with the accessions NI271, NI276 and NI356.

Keywords: Cucurbitaceae, anther culture, thermal shock, androgenesis, plant regeneration.

INTRODUCTION

The cucurbits are among the first plant species domesticated by humans (Bisognin et al., 2002). On the economic front, Cucurbitaceae family is the second important horticultural plants after the Solanaceae (Esteras et al., 2012). Some cultivars of this species are called "*Egusi*" in Benin, Nigeria, and "*pistache*" in Côte d'Ivoire. These cucurbits are prized for their oilseeds and are

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consumed as soup thickener. The seeds are good sources of lipids and proteins (Loukou et al., 2007). In West Africa, particularly in Côte d'Ivoire, cucurbits have a great socio-economic interest, due to nutritional and therapeutic properties (Azi, 2013; Dhiman et al., 2012). Cucurbits have numerous agronomic potentials. They are well adapted to extremely divergent agroecosystems and to various cropping systems. They are also characterized by low-input agriculture (IPGRI, 2002; Yousif et al., 2011). Increasing production and uses of these cucurbits can contribute in food security and diversify small farmers income (Williams and Haq, 2002**)**.

Surveys and collecting missions conducted in Côte d'Ivoire revealed the existence of five species of oleaginous cucurbits (Zoro Bi et al., 2003). Among all these species, *L. siceraria* has the best potential of productivity but the seed yields are remained low (Zoro Bi et al., 2003; Achigan et al., 2006).

To improve the productivity of this species, several studies have been conducted in Côte d'Ivoire. These studies concern the agronomical performances of oleaginous curcubit in fields allocated to woman and the selection of best genotypes expressing high seed yield. The creation of genotypes with good agronomic characteristics requires several rounds of selection, and thus many years of efforts (Datta, 2005)**.** The regeneration of haploid plants during cultivation of pollen grains has opened new perspectives to shorten the time of homozygous lines. This technique has become one of the major sources of haploid plant production used to develop homozygous diploids in plant breeding programs (Hofer et al., 2008; Jia et al., 2014). Nevertheless, there are a number of factors that affect androgenesis, including genotype, physiological state of the donor plant (Kumar et al., 2003; Qi et al., 2011; Chen et al., 2013), anther age, pollen developmental stage, pre-culture treatment (Devaux and Pickering, 2005; Koleva-guedeva, 2007), temperature, light, atmospheric condition, anther density, chemical factor such as culture medium, sugar and plant growth regulator (Koleva-guedeva, 2007; Ayed et al., 2010). Pollen irradiation (UV, gamma rays, and X-rays) is the most widely used technique to induce *in situ* parthenogenetic haploid plants of cucurbit. Induction of *in situ* haploid embryos and regeneration of *in vitro* haploid plants have been achieved using an irradiated pollen technique in *Cucurbita maxima* Duchesne (Kurta and Balkaya, 2010)**,** in *Cucumis melo* var. momordica (Godble and Murthy, 2012). There are no reports on induction of haploids in *L. siceraria*, so, the aim of this research was to develop a protocol for *in vitro* culture of anthers in order to shorten the time to obtain pure lines of *L. siceraria*. Investigations concern the determination of factors, which influence *L. siceraria* anther culture in order to elaborate an efficient method of haploid production. The parameters investigated were, effect of culture media and thermal choc on anthers for callus induction.

MATERIALS AND METHODS

Plants materials

Six accessions of *Lagenaria siceraria*, originated from Côte d'Ivoire were used in this study: cultivars NI 276 and NI 356 come from North-East, NI 185 from East, while NI 215 and NI 202 come from the South of Côte d'Ivoire. An accession is a sample collected in one field or obtained from one farmer's stock. The origin of the cultivar NI 271 is not known. Flower buds with microspores were collected from 28 to 35 day-old healthy plants cultivated, between the months of July and August 2012, under greenhouse of the University of Liège Gembloux Agro-Bio Tech (GxABT). The mean temperature, photoperiod and relative humidity during anther donor plant cultivation were 27±2 °C, 16 H and 75%, respectively.

Histological studies

The developmental stage of the microspores of each cultivar was determined by microscopic observation. Anthers were divided into four classes according to their size:

- < 5 mm corresponding to closed flower buds stage;
- 5 – 7 mm corresponding to the opened sepals flower stage;
- 7 – 9 mm and 9 – 11 mm representing the stages of small and large white buds, respectively.

Once collected, the buds were fixed in formalin-100% acetic acid-70% ethanol (5:5:90, v/v/v) and later stored in 70% ethanol at 4 °C in the dark. After fixation, the samples were dried in a mineral water + alcohol solutions with an increasing concentration of ethyl alcohol and then immersed in a mixture of pure resin and of absolute ethanol for a period of 24 hrs. Samples were finally dipped in a resin solution for hardening. After this stage, hardened samples were coated in histoblocs filled with syrupy mixture for histological sections. The sections were cut with a rotary microtome (Micron H360). Then, they were stained with Toluidine Blue solution [Sigma T3260] and dried in an oven at 35 °C. Stained sections were mounted between slide and cover slip. The observation of gametogenesis stages was made using a light microscope Nikon Eclipse brand LV-150.

Thermal pre-treatment of flower buds

To study the effect of cold treatments on callus induction from anthers, several durations of treatment at 4 °C were tested. Flower buds were collected from healthy plants between 9:00 and 10:00 a.m. and kept in a beaker containing 10 ml of tap water. The mouth of the beaker was tightly wrapped with aluminum foil to maintain a high humidity and kept in dark at 4 °C for six different durations (0, 1, 2, 4, 6, 7 days). For each accession, 21 flower buds (63 anthers; with 3 anthers per bud) were used for each pre-treatment duration.

Anthers culture conditions

Before the anthers culture, flower buds were sterilized under a horizontal laminar air flow. Four disinfection protocols were tested for the anthers surface sterilization (Table 1).

For each treatment, one or two drops of Tween 20 were added and between two consecutive steps, in each protocol, explants were rinsed three times with sterile distilled water for 3 min.

Two protocols developed by Metwally et al. (1998) and Kumar et al. (2003) were used for callus induction. Metwally et al. (1998) used Murashige & Skoog basal medium (1962) (Table 2). The Gamborg basal medium (B5) (Gamborg et al., 1968) is used by Kumar et al. (2003) (Table 3). The pH of media was adjusted to 5.7 before autoclaving. Approximately, 20 ml of medium was dispensed into each Petri dish. After sterilization, the anthers with size 5 - 7 mm, supposed to contain uni-nucleate microspore according to the histological study described above, were placed on medium and incubated in total darkness in a growth chamber at 24 ± 2 °C, 75% relative humidity (RH) for 2 weeks. Seven Petri dishes containing nine anthers per dish were incubated for each treatment and each accession. After this period, anthers were transferred in a growth chamber at 24 ± 2 °C, 75% RH, and illuminated with cool-white fluorescent lights (PPFD: 25 $\mu mol.m^{-2}.s^{-1}$) under a 16/8 h (day/night) photoperiod for two weeks.

Data collection and analysis

A complete randomized design was used for all experiments. For each treatment, 63 anthers were cultured (9 anthers per Petri dish and 7 replicates per treatment and per accession). The cultures were observed periodically and morphological changes were recorded at regular intervals. The number of anthers inducing callus was counted and the results were expressed as percentage of anther per each treatment and duration of pre-treatment. The data were subjected to analysis of variance (ANOVA). For significant effects, we used multiple comparisons of means (LS means statement of GLM procedure with a level of significance of 0.05) to identify the

best percentage of anther per each treatment and duration of pre-treatment. All statistical analyzes were achieved using Minitab® statistical package for Windows, version 16.

RESULTS AND DISCUSSION

Flower buds disinfection

The fourth protocol characterized by flower buds washing in ethanol 70% for 30 min and then in sodium hypochlorite 5% for 3 min prevents 100% infestations, regardless accession and duration of pre-treatment. This protocol is more efficient for flower buds disinfection, contrary to the three other methods that induced more than 50% of infection.

Microsporogenesis and anthers size

Microscopic observation after transversal and longitudinal sections indicates three cytological stages, according to anthers size: tetrad, uninucleate microspores and binucleate microspores (Figure 1). Results showed that when anthers size is inferior to 5 mm, tetrad stage was observed with all the accession (Figures 2A and 2B). Uni-nucleate stage occurred both in class 5 – 7 mm and in class 7 – 9 mm (Figure 2C). However, more than 90% of these microspores were in the class 5 – 7 mm. In class 9 – 11 mm, all microspores are bi-nucleate (Figure 2D).

The process of microspore formation occurs in a chronological order and could be correlated with floral bud size (Goldberg et al., 1993). According to the works of Kumar et al. (2003) on *Cucumis sativus* L, it appears that the optimum sampling point is when the young microspore uni-nucleate will enter mitosis. Similarly, results were obtained with others plants such as barley (Hoekstras et al., 1992) and rice (Swapan, 1990). Given that uni-nucleate stage is the best for anthers culture, the class 5 – 7 mm has been chosen for callus culture (Figure 1).

Callus induction

The nuclear stage of the microspore is a key factor for callus induction and subsequent embryos formation (Palmer and Keller, 2005). In the present study, anthers cultured on MS basal medium (M1, M2, M3, and M4) increased in volume, but did not expressed callus induction and subsequently turned brown and became necrotic after 4 weeks of incubation, regardless the accession and duration of pre-treatment. The oxidation of the anthers wall inhibits the callus formation (Qi et al., 2011; Custodio et al., 2005; Junqiang et al., 2008). This problem could not be circumvented by transfer of anthers onto fresh medium whenever exudation was observed, or by the use of antioxidants (data not shown). The inhibitory effect of browning on callus was also observed in anther cultures of *Fagus sylvatica* L (Jorgensen et al., 1997). However, basal medium B5 supplemented with 2,4-D, BAP and some amino acids such as arginine, glutamine and glycine promotes, 12 days after anther incubation, callus induction with a rate varying between 37 and 91% among the accessions (Table 4). Callus clumps exhibited prominent structures with smooth surfaces which are proembryos (Figure 3). In this study, callus induction has been improved with the addition of amino acids and cytokinin to the induction medium. In *C. sativus*, addition of a lower concentration of cytokinins to B5 basal medium, promotes the induction of callus and embryogenesis. The combination of 2,4-D with benzyl adenine was found superior to kinetine and thidiazuron as it induced the highest number of embryos (Kumar et al., 2003). Cytokinins are involved in protein synthesis. Their addition to the culture medium should accelerate cellular metabolism (Ziv et al., 2007). This callus induction is strongly influenced by the duration of cold treatment and by the accession (Table 5). There is also a significant interaction between the accessions and duration of treatment for the callus induction. Indeed, 100% of anthers from NI271, NI276 and NI356 produced callus when they are pretreated for two days at 4 °C (Table 6). The best percentages of callus induction, 79.06 ± 17% and 79.84 ± 22%, were obtained when the buds are pretreated for one and two days, respectively, at 4 °C. The percentage of callus induction was significantly reduced with treatment durations higher than four days (Table 6). However, accessions (NI276 and NI356) from North-Eastern Côte d'Ivoire have

the highest percentages of callus induction compared to those from the South and East (NI202, NI215 and NI185).

A number of factors influence callus induction and embryogenic response of cultured anthers and microspores and these have been extensively reviewed (Rodeva et al., 2004; Zhang et al., 2011). For some species and genotypes, these requirements may be more stringent than for others. Application of cold and heat stress pre-treatment has been an essential factor to increase the efficiency of androgenesis in different species (Pechan and Smykal, 2001). For *L. sciceraria*, two days cold pre-treatment (4 °C) proved to be optimal as it gave the best callus induction. Similarly, in *C. sativus* the cold stress pre-treatment (4 °C for 4 days) increases callus and embryos induction (Kumar et al., 2003). However, some species give the highest percentage of callus induction when anthers are pre-treated with heat. For example, *Phoenix dactylifera* L. induces the best percentages of callus when flower buds are pre-treated at 38 °C for seven days (Chaibi et al., 2002). The optimum temperature and duration of pre-treatment vary from one species to another. The mechanism of temperature shock in inducing higher androgenesis is not well known. One of the possibilities suggested is the reduction of ABA levels in the cultured anthers (Srivastava and chaturvedi, 2008). Cold and heat pre-treatment could also disrupt the cytoskeleton of the microspores which predispose the induction of callus and embryogenesis.

Table 1: Protocols of flower buds disinfection.

Protocols	Flower buds disinfection	
1	Ethanol 70% for 2 min	Sodium hypochlorite 5% for 20 min
2	Ethanol 70% for 1 min	Sodium hypochlorite 5% for 10 min
3	Ethanol 70% for 30 min	Sodium hypochlorite 5% for 5 min
4	Ethanol 70% for 30 min	Sodium hypochlorite 5% for 3 min

Tables 2: Media of callus induction of *Lagenaria siceraria* (Molina) Stand, inspired of Metwally et al. (1998) protocol.

Media	Sugar (sucrose) (g/l)	Hormone 2, 4-D (g/l)	Agar (g/l)
Medium 1	150	0.005	8
Medium 2	150	0.0025	8
Medium 3	90	0.005	8
Medium 4	90	0.0025	8

Table 3: Composition of medium used for callus induction in *Lagenaria siceraria* (Molina) Stand, according to Kumar et al. (2003) protocol**.**

Medium	Sucrose (g/l)	Agar (g/l)	2,4-D (g/l)	BAP (g/l)	Amino acids (g/l)
Gamborg basal medium (B5)	85.5	8	$8.84.10^{-4}$	$4.5.10^{-4}$	Cysteine: 0.121 Arginine: 0.174 Glutamine: 0.146 Glycine: 0.075

Table 4: Accessions effect on the percentage of callus induction from anther *Lagenaria siceraria* (Molina) Standl grown on Gamborg basal medium.

Accessions Number	Accessions origin	Percentage of callus induced
NI185	Est	55.04±21.65^{c}
NI202	South	49.78±12.60^{d}
NI215	South	37.21±10.42^{e}
NI271	-	91.72±7.35^{a}
NI276	North-Est	82.91±10.14^{b}
NI356	North-Est	81.92±13.82^{b}
	F	24836.26
	P	<0.001

Mean within a column followed by the same letters are not significantly different at $p < 0.05$.

Table 5: Effect of the interaction pre-treatment duration-accession of *Lagenaria siceraria* (Molina) Standl on the percentage of callus induced.

Accessions	Durations of thermal treatment	Percentage of callus induced
NI185	0	49.20±21.53^{r}
	1	89.10±10.23^{f}
	2	75.21±18.02^{j}
	4	39.12±09.98^{v}
	6	52.53±24.07^{q}
	7	25.10±20.62^{x}
NI202	0	30.58±17.21^{w}
	1	60.62±18.22^{o}
	2	63.67±17.51^{m}
	4	39.29±20.24^{tv}
	6	60.18±19.81^{o}
	7	44.33±18.42^{s}
NI215	0	30.14±21.23^{w}
	1	54.33±20.12^{p}
	2	40.19±18.47^{t}
	4	31.00±18.21^{w}
	6	44.29±17.52^{s}
	7	23.29±20.12^{y}
NI271	0	81.04±12.23^{h}
	1	100.00±00.00^{a}
	2	100.00±00.00^{a}
	4	94.92±6.32^{b}
	6	89.31±10.21^{ef}
	7	85.03±10.42^{g}
NI276	0	69.99±17.67^{l}
	1	76.36±16.74^{i}
	2	100.00±00.00^{a}

	4	90.41±7.34^{d}
	6	84.34±11.02^{g}
	7	76.39±20.12^{i}
NI356	0	73.06±21.35^{k}
	1	93.96±7.32^{c}
	2	100.00±00.00^{a}
	4	72.72±20.41^{k}
	6	90.17±7.48^{de}
	7	61.64±20.56^{n}
F		765
P		<0.001

Mean within a column followed by the same letters are not significantly different at $p < 0.05$

Table 6: Effect of the duration of cold treatment (4° C) on the percentage of callus induced with the anthers of *Lagenaria siceraria* (Molina) Standl., considering all accessions.

Duration of thermal pre-treatment (days)	Percentage of callus induced
0	55.67±20.52^{cd}
1	79.06±17.11^{a}
2	79.84±22.86^{a}
4	61.24±26.08^{c}
6	70.14±18.68^{b}
7	52.63±24.01^{d}
F	7066.70
P	<0.001

Mean within a column followed by the same letters are not significantly different at $p < 0.05$.

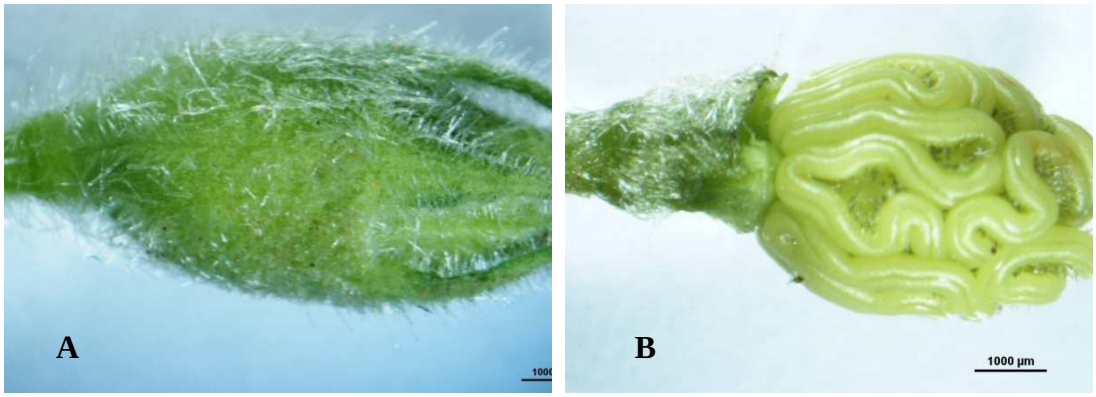

Figure 1: Floral bud of *Lagenaria siceraria* (Molina) Standl at open sepals stage**;** Anthers color is green and contain microspores at uninucleate. A: Flower bud with perianth, B: Flower bud without perianth.

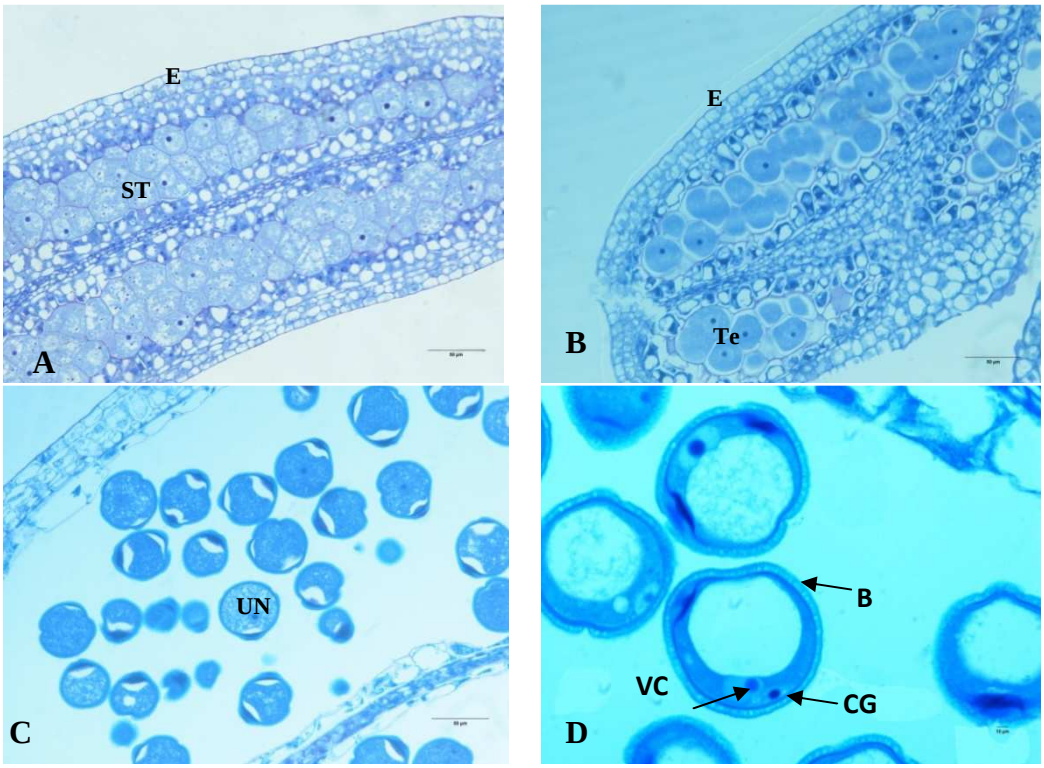

Figure 2: Development stages of pollen grains of *Lagenaria siceraria* (Molina) Standl. A & B: Young pollens from flower bud less than 5 mm (E = epidermis, ST = sporogenous tissues, Te = Tetrad); C & D: Pollens from anthers size 5 – 7 mm (UN = Uni-nucleate microspores released in pollinic sac; B = Bi-nucleate stage with Cell Generator-CG and Vegetative Cell-VC) (bar = 10µm).

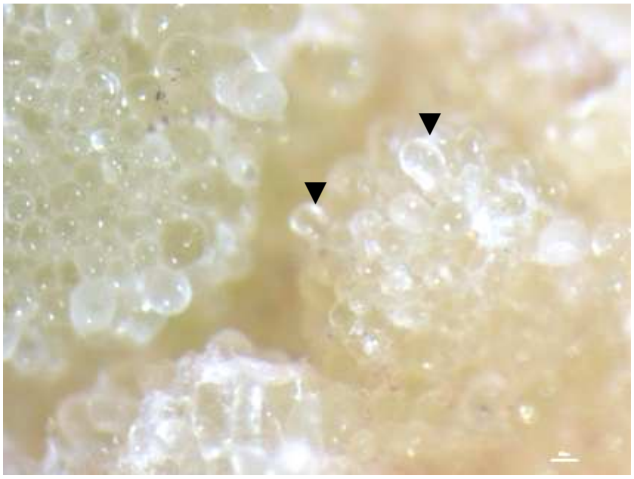

Figure 3: Embryogenic calluses with proembryos (the arrowhead indicates the protuberant structures with smooth surfaces) (Bar = 100µm).

Conclusion

Determining the anthers length in relation to the development of microspores, showed that anthers size between 5 and 7 mm corresponding to the uni-nucleate stage, is more appropriated to culture. Therefore, this phenological criteria may be used in *L. siceraria* androgenesis. Our results have showed that the genotype or accessions might play more decisive role in callus induction. The thermal shock 4 °C for 2 and 4 days is necessary to stimulate callus production. The basal medium B5 modified with 2,4-D, BAP and some amino acids is identified as the efficient medium for callus induction.

From these results, it is possible to envisage the production of haploid plants through anther culture in *L. siceraria*. However, Further research need to be made to show that callus obtained do not come from maternal tissue surrounding pollen and callus ploidy must be investigated.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

KLK and TSD conducted the studies and drafted the manuscript; KKK and THK contributed to manuscript writing; KIK did the statistical analysis; IAZB was the project coordinator; J-PB contributed to manuscript writing and was the project supervisor.

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