Determination of a Suitable Protocol for Indigenous Oilseed Cucurbits Plant Regeneration

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Summary

The present work was carried out to establish rapid in vitro *propagation of the indigenous oilseed* Citrullus lanatus. *Efficiency of 3 protocols for seed decontamination was investigated. High level of seed sterilization was obtained after removing seed coat and soaking the seeds in 1.6% sodium hypochlorite with a drop of Tween 20. Shoot tips, single node and cotyledon explants of 3 morphotypes have been screened for adventitious shoot formation in tissue culture. Best response in terms of multiple shoot induction was obtained from cotyledon proximal part with hypocotyl segment on Murashige and Skoog medium supplemented with 1 mg/l 6-Benzylaminopurine, 30 g/l sucrose and 8 g/l agar. After 3 weeks of culture, 90% of cotyledon proximal parts induced shoot. An average of 12.6 shoots per explant and a mean shoot length of 8 mm were obtained after multiplication stage. Shoot induction appeared to be strongly influenced by genotype and explant type. The percentage of shoot induction from cotyledon proximal parts ranged from 23.3% to 64.0% according to the genotype. Efficient rooting was achieved on half-solid MS medium containing 0.1 mg/l 1-Naphthaleneactic acid or without growth regulator. Acclimatised plantlets were transferred to greenhouse where they grew and flowered like seeded plantlets.*

Résumé

Détermination d'un protocole adéquat pour la régénération de la cucurbite oléagineuse indigène

Le présent travail est mené pour mettre au point un protocole efficace de régénération in vitro *pour la cucurbite oléagineuse indigène* Citrullus lanatus. *L'efficacité de 3 protocoles pour la désinfection des graines est étudiée. L'élimination des contaminants est obtenue lorsque les graines décortiquées sont trempées dans 1,6% d'hypochlorite de sodium additionné d'une goutte de Tween-20 durant 25 mn. Les bourgeons apicaux, les nœuds et les cotylédons de trois morphotypes sont utilisés pour l'induction de pousses adventives. La meilleure réponse pour l'induction de pousses est obtenue avec les parties proximales de cotylédons sur le milieu de Murashige et Shoog complétés avec 1 mg/l 6-Benzylaminopurine, 30 g/l de saccharose et 8 g/l d'agar. Après trois semaines de culture, 90% des parties proximales de cotylédon ont induit des pousses. Une moyenne de 12,6 pousses par explant avec une longueur moyenne de 8 mm est obtenue après le stade de multiplication. L'induction de pousses, fortement influencée par le génotype et le type d'explant, varie de 23,3% à 64,0% selon le génotype. Les plantules ont pu s'enraciner sur le milieu MS semi-solide contenant 0,1 mg/l d'acide 1-Naphthaleneactique ou sans hormone. Les plantules acclimatées sont transférées en serre où elles se sont développées et ont fleuri normalement.*

Introduction

Cucurbitaceae is an important family of vegetables grown worldwide. According to their mode of consumption, two major groups are distinguished: the type consumed fresh or in salads (watermelon, melon, squash, etc.), and the type grown for their seeds (bitter cucumber, African melon, etc.) (43). In contrast to the first group that is well documented (9, 23, 34), little is known about the second (24, 35, 42). The indigenous edible-seeded cucurbits are classified into minor crops. Species cultivated in Ivory Coast are *Cucumis melo* var. *agrestis* (Naudin), *Citrullus lanatus* (Thunb.) Maktum and Nakai, *Cucumeropsis mannii* (Naudin) and *Lagenaria siceraria* (Molina) Standl (42). They are widely used in western and eastern Africa for their numerous agronomic, medicinal and economic values (14). Their dried seeds designated "pistachio" in Ivory Coast (32), and "egusi" in Nigeria, Benin, Cameroon and Congo (20, 35), are slightly toasted, ground and used as soup thickener (43). Edible oil can also be extracted from the seeds (4, 39). It should be noted that "egusi" is a generic name referring to all the *Cucurbitaceae* with edible seeds (1).

Species *C. lanatus* was subdivided in three sub-species by Fursa (17): ssp. *vulgaris* (Schrad.) Fursa;

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ssp. *lanatus* (Thunb) Matsum. and Nakai; ssp. *mucosospermus* (Fursa). Indigenous oilseed *C. lanatus* is classified in ssp. *mucosospermus* contrary to watermelon (*C. lanatus*) classified in ssp. *lanatus* (17). Studies are in progress to identify the botanical name of indigenous oilseed *C. lanatus*. Meanwhile we will designate indigenous oilseed as *C. lanatus*.

Considering the great morphological variability of indigenous oilseed *C. lanatus* cultivated in Ivory Coast, Zoro Bi *et al.* (43) described two different cultigroups. The term cultigroup was first used by Westphal (40) with a view to adapt the plant nomenclature to the classification of the cultivated plants. The first cultigroup, containing three cultivars defined on the basis of averaged seed size, has smooth seeds that are tapered to the point of attachment. The second cultigroup represented by one cultivar has ovoid and flattened seeds, with a thick and rough margin. Only the first cultigroup was used in the present study.

Multiplication of the indigenous oilseed cucurbits can be achieved through botanical seeds but the maintenance of appropriate cultivar is a major problem due to their cross-pollinating breeding system. In addition, seeds loose quickly their germinating capacity that cannot be preserved more than one year depending upon storage conditions (14). Yet, to satisfy the future needs in genetic resources, it is imperative to collect and conserve representative stocks of plant genetic diversity (19). Traditional production systems of indigenous oilseed cucurbits should be improved with the aim of developing new economic resources for local communities. Such an approach would thus contribute effectively to poverty and rural exodus reduction. In this context, an effective protocol for indigenous oilseed cucurbits micropropagation should be developed to meet several purposes: germplasm conservation, multiplication of elite improved cultivars and distribution of plant material. There have been reports on micropropagation in species of *Cucurbitaceae* cultivated for their fruits such as winter squash (*Cucurbita maxima* Duch.) (27), summer squash (*Cucurbita pepo* L.) (3), cucumber (*Cucumis sativus* L.) (34), melon (*Cucumis melo* L.) (23), dessert watermelon (*Citrullus lanatus*) (9) and bottle gourd (*Lagenaria siceraria*) (21). However, such investigations were not carried out with indigenous edible-seeded cucurbits. The genotypes have been shown to greatly influence plant regeneration (2). Genotype-specific responses have been documented in *Cucurbitaceae* family (28) as in others family: *Ericaceae*, *Fabaceae*, *Rosaceae*, etc. (5, 13, 16). This specific response necessitates the determination of a suitable protocol to stimulate plant regeneration in genotypes of interest. Several types of explants (shoot tips, single nodes, cotyledons, hypocotyl and root) were used for cucurbits micropropagation (6, 10, 26, 27). All reports of *Cucurbitaceae* shoot organogenesis (6, 9, 15, 36) involved the use of media formulated with the basic macro and micro salts plus vitamins as outlined by Murashige and Skoog (30). Optimum shoot regeneration has been achieved generally by using 6-Benzylaminopurine (BAP) as the only plant growth regulator (6, 7, 9, 36). However, combination of Indole-3-butyric acid (IBA), kinetin and Gibberellic acid (GA_3) has been successfully tested for direct shoots induction by Compton *et al.* (10). Shoot elongation was improved by using kinetin or without growth regulator (7, 15). Rooting was achieved with IBA, 1-Naphthaleneactic acid (NAA) and Indole-3-acetic acid (IAA) or without growth regulator (6, 10, 26, 27).

This study is aimed to determine an efficient protocol for *in vitro* regeneration of edible-seeded cucurbits. It is a prerequisite to develop programmes for the conservation and large-scale *in vitro* propagation of desired genotypes of indigenous edible-seeded cucurbits. Particularly, the objectives of the present work are to: (i) identify an effective seed disinfection protocol; (ii) identify optima media, explant type, seedling age for indigenous edible-seeded cucurbits micropropagation and (iii) investigate the influence of genotype on shoot induction.

Material and methods

Plant material

Mature seeds of three genotypes of indigenous oilseed *Citrullus lanatus* were used as explant source. The three genotypes were designated as follows: CL for genotype with large seeds (average of 120 mm^2), CM for genotype with medium seeds (average of 59 mm^2), and CS for genotype with small seeds (average of 42 mm^2). All these genotypes were selected from a germplasm collection at the University of Abobo-Adjamé (UAA) Abidjan, Ivory Coast. The genotypes CL, CM, and CS were collected respectively in three different ecological regions of the country: the southern zone (tropical rain forest), the central zone (woodland savannah with some herbaceous areas) and the eastern zone (transitional woodland savannah with blocks of semi-deciduous forests).

Sterilization

To establish a performing technique of decontamination, we tested only the genotype CM. Three protocols of decontamination described by Jaskani *et al.* (22), Nasr *et al.* (31) and Tang *et al.* (38) have been compared. After the preliminary tests (data not show), the three protocols were improved by an increase of the concentration of sodium hypochlorite from 1% and 1.3% to 1.6%. In the first and second protocol seeds with coat were treated respectively with 70% alcohol for 30 s and with 20% hydrochloric acid (HCl) for 20 min. Then, the seeds from the two treatments were surface-sterilized in a 1.6% sodium hypochlorite solution containing 15 g/l of active chlorine, with one

drop of Tween 20 for 15 min and 20 min for the first and the second protocol, respectively. In the third protocol, seed coats were removed manually, and the embryos surface-sterilized 25 min in a 1.6% sodium hypochlorite with one drop of Tween 20. The treated seeds from the three protocols of decontamination were rinsed six times with sterile distilled water. Decontaminated seeds from each protocol were sown in petri-dishes containing 20 ml MS basal medium (30) without growth regulators. The pH of media was adjusted to 5.7 before autoclaving at 121 °C for 20 min. Each petri-dish with 5 seeds constituted one replicate and there were five replicates per treatment. For the seed germination and seedling growth, the following climatic conditions were applied: 28 ± 2 °C with a 12h/12h light/dark cycle; light was provided by cool white fluorescent lamps with an intensity of 50 mol/ m^2/s. Germination and contamination was recorded daily during a week. Radicle emergency served as an indication of germination.

Preparation of the different explants

Eight explant types were used for micropropagation: proximal parts of the cotyledons with hypocotyl segment, distal parts of the cotyledons, shoot tips and single nodes from ten-, fifteen- and twenty-day-old seedlings. Preparation of those explants was carried out as follows.

Seeds of the three genotypes CL, CM and CS were sterilized with the best decontamination protocol. In order to reach full development of the seedlings, seeds were germinated in jars containing MS basal medium without growth regulators, during 10, 15 and 20 days in climatic conditions described above. However, with the 5-day-old seedlings, seeds were germinated in darkness. This condition for 5-day-old seedlings was chosen on the basis of investigations carried out by Compton (6). Germinating embryos in darkness improved shoot organogenesis from cotyledons.

Cotyledonary explants were removed from 5-day-old seedlings as follows. The hypocotyl was first cut off close to the cotyledons, and then the cotyledons were cut in half resulting in the proximal and distal parts (Figure 1a). Subsequently each part (distal parts and proximal parts of the cotyledons with hypocotyl segment) was separated with a scalpel blade. The apical bud of the seedling on the proximal part was removed with care. This resulted in cotyledon distal and proximal parts with hypocotyl segment explants (Figure 1b).

In addition, shoot tips and single nodes were dissected from ten-, fifteen- and twenty-day-old seedlings and were also used as explants.

The shoot has been removed. 'Hyp' marks the part of the hypocotyl remaining after explant preparation. The cotyledon remnant is labeled 'Cot'.

Culture establishment

To determine the organogenetic responsiveness of different explant types at various ages, two sets of comparisons were made. The effect of seedling age on shoot induction was tested using several explant types of the genotype CM. Each explant type was cultured on induction media, i.e. MS medium containing four combinations of growth regulators and agar (Table 1). The media combination was selected on the basis of investigations carried out on *Cucurbitaceae* cultivated for their pulp (6, 10, 26, 27). Once identified the best performing factors (medium combination, the best cotyledon explants and the best day-old seedlings for shoot tips and single node dissection) for shoot induction, the genotypic influence was investigated. This experiment was performed with the three genotypes CL, CM and CS.

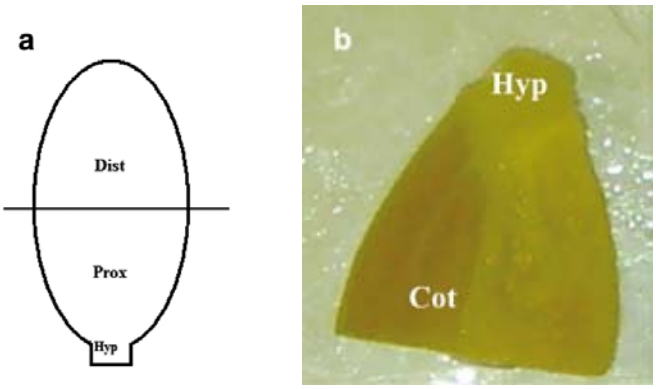

Figure 1: Adventitious shoots induction from de-budded cotyledon proximal part with hypocotyl segment explants.
a Preparation of explant from indigenous oilseed *Citrullus lanatus* cotyledons. The distal part (Dist) and proximal part (Prox) were cut from the cotyledons as indicated by the line. **b** Initial explant from the cotyledon proximal part of a 5-day-old indigenous oilseed *Citrullus lanatus* seedling.

After 3 weeks the shoots were subcultured on the same medium except for the medium M_{m2} where IBA, GA_3 and Kinetin were substituted for 1 mg/l BAP. In each medium, shoot multiplication was scored after 3 weeks. A shoot consisted of a shoot apex, an elongating stem (≥ 4.0 mm long) and expanding leaves with petioles.

Shoots harvested from multiplication stage were transferred to shoot elongation media (Table 1) during 2 weeks. All media tested contain the growth regulator BAP at different concentrations or no growth regulators. Shoots were then placed in 150 mm × 20 mm culture tubes containing 15 ml of rooting media, i.e. MS medium containing various auxins or without growth regulators (Table 1). The hormone-free media differ in their agar amount.

Explants were cultured in 55 mm × 70 mm glass flasks, each containing 25 ml of medium. Each medium combination consisted of twelve replicates, one replicate being one flask with five explants. After induction and multiplication stage, explants were scored for shoot induction, number of shoots and

Table 1
Media tested for indigenous oilseed *Citrullus lanatus* shoots induction, shoots elongation and roots induction. All media tested were formulated with the basic macro and micro salts plus vitamins as outlined by Murashige and Skoog (MS) (1962) and supplemented with 30 g/l sucrose

	Induction				Elongation				Rooting			
Compounds	M_{i1}	M_{i2}	M_{i3}	M_{i4}	M_{e1}	M_{e2}	M_{e3}	M_{e4}	M_{r1}	M_{r2}	M_{r3}	M_{r4}
Basal medium	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS
BAP (mg/l)	0.5		1	2			0.1	2				
IBA (mg/l)		0.35										0.1
GA_3 (mg/l)		0.1										
Kinetin (mg/l)		0.1										
NAA (mg/l)										0.1		
Agar (g/l)	7	5	8	8	7	5	8	8	7	5	4	8

shoot length. For elongation stage shoot length was evaluated and for rooting stage the number of shoots with roots was recorded.

Acclimatization

After two weeks on the rooting medium, the plantlets were transplanted into plastic pots filled with autoclaved soil [80% of Klasmann® 4 Special N° 26, 15% peat, 5% of the Rhine sand and organic fertilizer (0.6 g/l mixture)], covered with a glass or a plastic lid and grown in a growth-room with the following parameters: 24° C/20° C day and night temperature, relative humidity ranging from 50 to 60%, 12h/12h light/dark cycle, and a light intensity of 170 µmol/m^2/s. The lid was gradually removed once a majority of the plantlets exhibited new growth (about two weeks); seedlings were then moved to the greenhouse. The acclimatization phase was considered successful when plantlets were adapted to *ex vitro* conditions and developed new leaves. The number of plantlets that survived acclimatization was recorded three weeks after transfer to the greenhouse.

Statistical analysis

The effect of different media was quantified and data was analyzed using standard analysis of variance (ANOVA). When the null hypothesis of an ANOVA was rejected, means were compared using Tukey's multiple range test at 5 % level of significance. Data were processed using the software Minitab ® for Windows, version 14.00. Data of single node explants from fifteen- and twenty-day-old seedlings were transformed using logarithmic transformation prior to analysis by standard analysis of variance (ANOVA) procedures (12).

Results

Sterilization

With the first and the second decontamination protocol, all the seeds were contaminated and no germination was recorded. The third decontamination protocol, for which seed coats were removed, resulted in the complete elimination of contaminants without affecting seed germination. After 7 days 100% of the seeds treated with the third decontamination protocol germinated. The third decontamination protocol (seed without coats were surface-sterilized 25 min in a 1.6% sodium hypochlorite with one drop of Tween 20) was therefore used in all subsequent procedures.

Shoots regeneration

Shoots differentiated directly after 3 weeks of culture for all explant types with no intermediate callus stage. Figure 1c shows an example of shoots induced on cotyledon proximal part with hypocotyl segment.
Most of explant types cultured on media M_{i1}, M_{i3} and M_{i4} which all contained BAP reacted more favourably to shoot induction than those placed on M_{i2} which did not contain BAP (Figure 2a). However, whatever the media, no shoots were observed with the explants made from the distal part of cotyledon and with shoot tips from 10-day-old seedlings. It was found that the best shoot induction response occurred in medium M_{i3} supplemented with 1 mg/l BAP when considering the percentage of shoots induction and the number of

Figure 1: Adventitious shoots induction from de-budded cotyledon proximal part with hypocotyl segment explants. **c** Explant after 3 weeks in culture onto MS medium supplemented with 1 mg/l BAP, 30 g/l sucrose and 8 g/l agar. Multiple shoots cover the area between the hypocotyl 'Hyp' and the cotyledon 'Cot'.

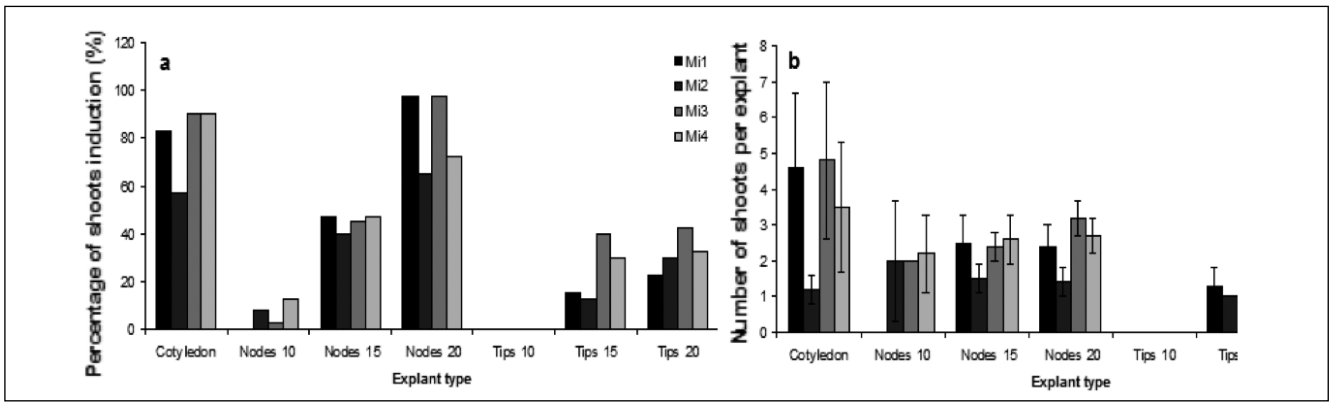

Figure 2: Influence of four media and explant types on shoots induction in indigenous oilseed *Citrullus lanatus*. **a** Percentage of shoots induction. **b** Number of shoots per explant. Media tested included M_{i1} (1 mg/l BAP + 7 g/l agar), M_{i2} (0.35 mg/l IBA + 0.1 mg/l GA_3 + 0.1 mg/l Kinetin + 5 g/l agar), M_{i3} (1 mg/l BAP + 8 g/l agar) and M_{i4} (2 mg/l BAP + 8 g/l agar). Each point represents mean ± SE.

shoots per explant (Figure 2b).

The explant type used for shoot induction also significantly influenced the number of shoots produced (Figure 2b). Indeed, with respect to explant type response to the media tested, highly significant difference in the number of induced shoots was noted between media for cotyledon proximal part with hypocotyl segment (F= 32.13; P< 0.001). The number of shoots per explant produced on medium M_{i2} was significantly lower than those obtained on the three other media. Similarly, for single nodes from 20-day-old seedlings, highly significant difference was noted in the number of shoots induced between different media (F= 14.70; P< 0.001). For the other explant types no difference was noted in the number of induced shoots between different media.

The percentage of shoot induction and the number of shoots produced per explant were influenced by the genotype according to explant type used (Figure 3). The percentage of shoot induction was higher for the genotypes CM and CL when cotyledon proximal part with hypocotyl segment was used as explant. For genotype CL the percentage of shoot induction was higher when single nodes were used as explant (Figure 3a). There was however no significant difference in the

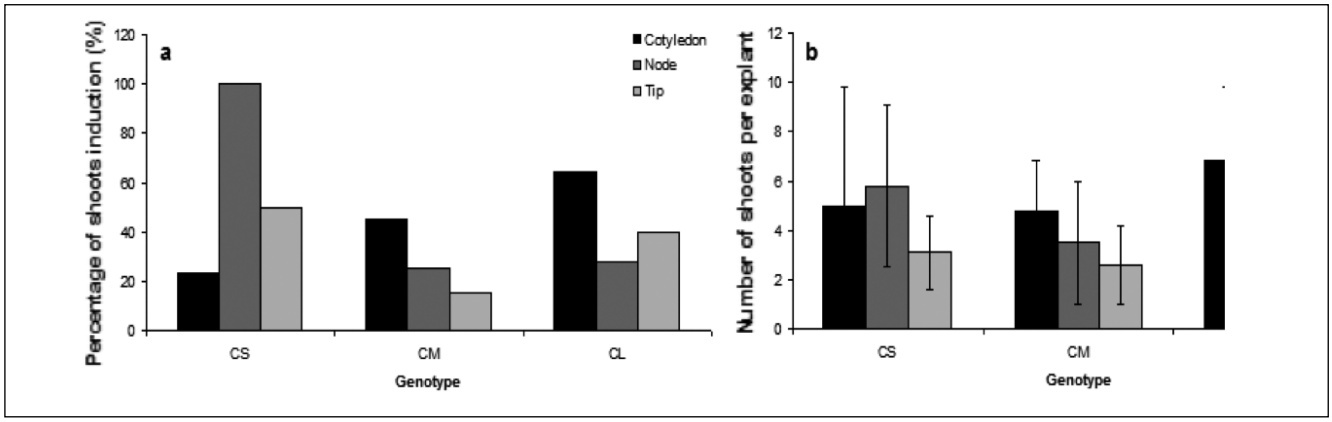

Figure 3: Effect of genotype and explant types on shoots induction in indigenous oilseed *Citrullus lanatus*. **a** Percentage of shoots induction. **b** Number of shoots per explant. Cotyledon proximal part with hypocotyl segment from 5-day-old seedlings, shoot tips and single node explants from 20-day-old seedlings were cultured onto MS medium supplemented with 1 mg/l BAP and 8 g/l agar. Each point represents mean ± SE.

Table 2

Effect of genotype and explant type on induced shoot growth of indigenous oilseed *Citrullus lanatus* genotypes. Cotyledon proximal part with hypocotyl segment from 5-day-old seedlings, shoot tips and single node explants from 20-day-old seedlings were cultured onto MS medium supplemented with 1 mg l^{-1} BAP and 8 g l^{-1} agar. Data represented as mean ± SE

	Shoot length (mm)		
Cultivar/Explant	Cotyledon	Single nodes	Shoot tip
CS	14.0 ± 8.4[a]	8.9 ± 2.3[a]	5.5 ± 1.0[a]
CM	14.2 ± 5.1[a]	7.4 ± 2.3[b]	5.8 ± 1.3[a]
CL	10.2 ± 5.5[a]	9.2 ± 3.0[a]	7.9 ± 1.9[b]
Probability (P)	0.139	0.005	< 0.001

Means in a column followed by a common letter are not significantly different at the 5% level (Tukey's multiple range test).

Table 3
Influence of medium composition during multiplication stage on the number of shoots per explant and shoot length. All media tested at this stage contained only BAP as growth regulator at different concentrations. Five shoots were cultured per vessel. Data represented as mean ± SE

Medium	Number of shoots per explant	Shoot length (mm)
M_{m1}	6.8 ± 4.7[b]	7.5 ± 1.3[b]
M_{m2}	8.6 ± 2.5[a]	9.4 ± 1.6[a]
M_{m3}	12.6 ± 5.3[a]	8.0 ± 1.4[b]
M_{m4}	6.4 ± 4.1[b]	6.8 ± 1.5[c]
Probability (*P*)	< 0.001	< 0.001

Means in a column followed by a common letter are not significantly different at the 5% level (Tukey's multiple range test).

number of shoots per explant and the shoot length between the tested genotypes for cotyledon proximal parts and for shoot tips (Figure 3b). Difference between the tested genotypes was only observed for single nodes (F= 3.40; P= 0.037 and F= 5.49; P= 0.005 respectively for number and length of shoots). Nevertheless, with all genotypes, the cotyledon proximal part with hypocotyl segment was the best explant type to produce the longest shoots (Table 2).

All media tested for multiplication stimulated shoot proliferation (Table 3). The average number of shoots per explant at this stage was superior to that obtained during shoot induction. For multiplication medium $M_{m2,}$ the average number of shoots per explant was sevenfold higher than the number of shoots observed at the induction stage. Highly significant differences in the number of shoots per explant were noted between multiplication media (F= 13.17; P< 0.001). The number of shoots per explant produced on medium M_{m3} was significantly higher than those obtained on media M_{m1} and M_{m4}, but did not differ from M_{m2}.

An increase in shoot length was noted in all elongation media (M_{e1}: 8.1 ± 4.7 mm, $M_{e2:}$ 19.9 ± 13.2 mm, M_{e3}: 9.7 ± 3.4 mm, M_{e4}: 7.7 ± 2.7 mm), particularly on medium M_{e2} for which the average shoot length was about two-fold superior to the values observed at the multiplication stage.

The regenerated shoots were transferred to rooting media for root induction. It was found that the best rooting response occurred in medium M_{r2} supplemented with 0.1 mg/l NAA after 2 weeks of culture (Figure 4).

Addition of NAA to the rooting medium M_{r2} improved shoot rooting. Indeed, the percentage of shoots that produced roots increased in medium M_{r2} compared to medium M_{r4} containing IBA or M_{r1} and M_{r3} without growth regulators. Plantlets obtained from shoots rooted in medium M_{r2} (Figure 5a) showed the highest percentage of acclimatization (31.6%). Those rooted in medium M_{r3} showed on the contrary the lowest acclimatization percentage (7.7%). Regenerated indigenous oilseed *Citrullus lanatus* plantlets were acclimatized (Figure 5b) and grew into normal plants in the greenhouse (Figure 5c).

Discussion

The first and the second decontamination protocols were not effective with the seeds of indigenous oilseed *Citrullus lanatus*. Yet, the same protocols have been used successfully on oil seed *Brassica* spp. L. and watermelon *Citrullus vulgaris* Schrad seeds (31, 38). Our results could be explained by the difficulty to eliminate microorganisms from the rough seed coats (not removed in the two first protocols). This hypothesis is plausible because the third decontamination protocol, for which seed coats were removed, resulted in the complete elimination of contaminants without affecting seed germination.

Combination of growth regulators has been reported to

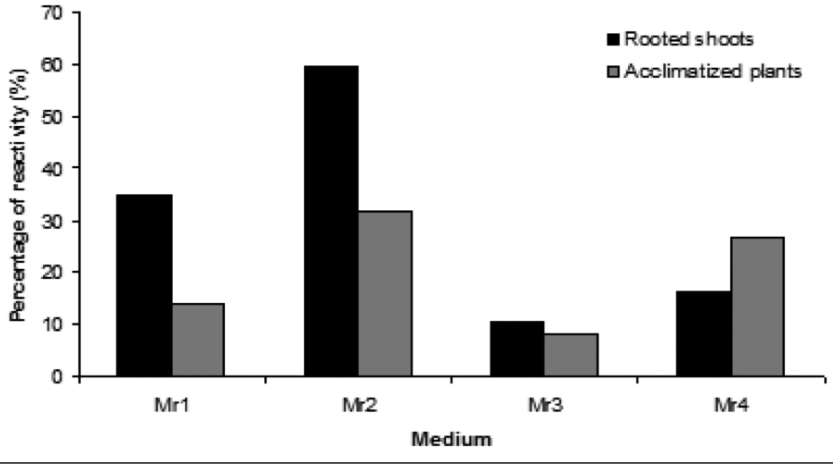

Figure 4: Influence of four media on the percentage of indigenous oilseed *Citrullus lanatus* shoots producing roots and on acclimatization percentage. Media tested included M_{r1} and M_{r3} without growth regulators but M_{r3} + 4 g/l agar, M_{r2} (0.1 mg/l ANA + 5 g/l agar) and M_{r4} (0.1 mg/l IBA + 8 g/l agar). Plantlets from each rooting medium were acclimatized. Percentage of reactivity is percentage relating to the number of rooted shoots and acclimatized seedlings.

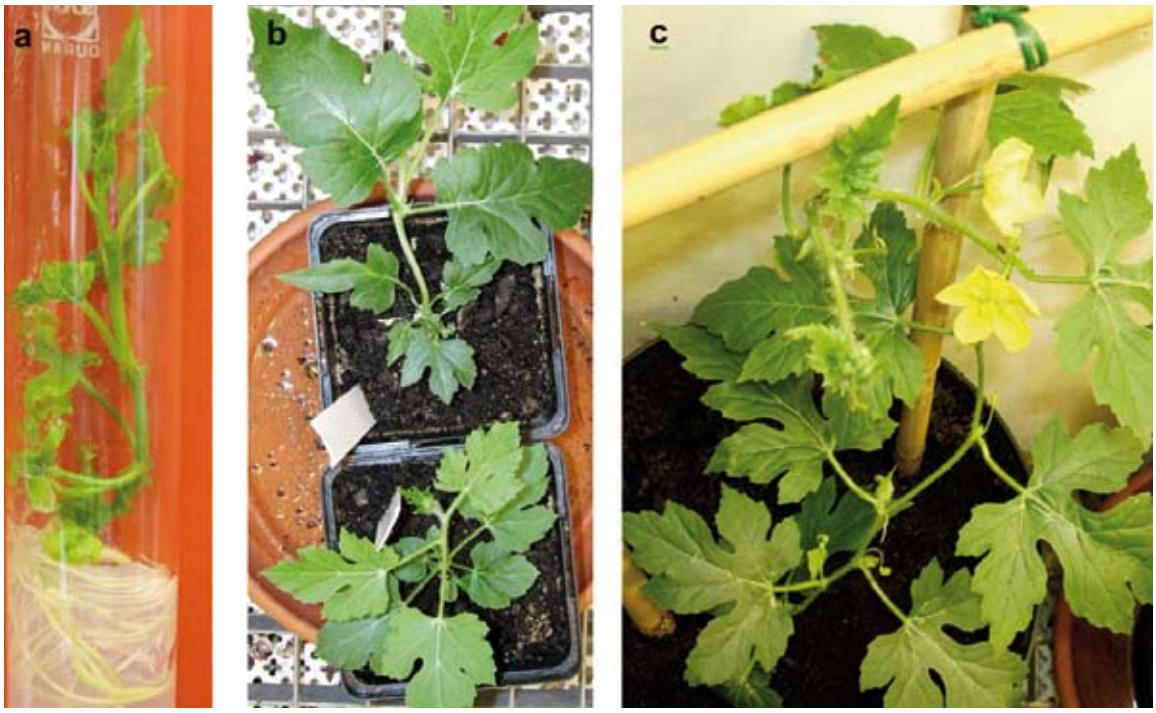

Figure 5: Plantlet regeneration in indigenous oilseed *Citrullus lanatus.*
a Rooted plantlets on M_{r2} (0.1 mg/l ANA + 5 g/l agar);
b Acclimatized plants in the greenhouse.
c Flowered acclimatized plant after 4 weeks of acclimatization.

determine the course of morphogenesis such as shoot organogenesis in cucurbits (10, 41). In our experiments, BAP was a crucial factor for the adventitious shoot induction and this was also reported by Srivastava *et al.* (36) and Dong and Jia (15) in *Citrullus vulgaris* and by Ntui *et al.* (33) in *Colocynthis citrullus* L. Indeed, induction medium M_{i2} without BAP but with IBA, GA_3 and Kinetin did not improve shoot induction. This could be due to the presence of GA_3 in the induction medium, since the addition of gibberellins to shoot induction media often reduces morphogenesis, according to George (18). However, shoot induction in micropropagation of *Cucumis hystrix* Chakr. has succeeded despite the addition of GA_3 to culture medium with IBA and Kinetin (10).

Previous studies have shown that seedling age from which explants are sampled can influence the regenerative response in *Citrullus lanatus* (8, 25), in *Momordica charantea* L. (37) and in *Lagenaria siceraria* (21). In the present study, shoot tips and single node from 20-day-old seedling showed higher percentage of regeneration than those from 10- and 15-day-old seedlings. This suggested that for indigenous oilseed *Citrullus lanatus*, an old seedling is required for shoot tips and single node samples. In contrast to our results, shoot tips and single node from 6 to 8-day-old seedlings of *Momordica charantea* reacted more favourably to shoot induction (37). Among explants derived from seedlings at the same age, single node showed generally a higher percentage of regeneration when compared with the shoot tip. Theses results were in accordance with the study of Sultana and Bari (37), indicating that the single node of *Momordica charantea* is more responsive than shoot tips. Only the cotyledon proximal part with hypocotyl segment produced shoots, while the cotyledon distal part did not produce any. Similar results were obtained by Ananthakrishnan *et al*. (3), Compton and Gray (7) and Curukc *et al*. (11).

The results indicated that adventitious shoot regeneration ability was strongly influenced by the genotype in indigenous oilseed cucurbits. Similar genotypic differences in responses for regeneration were observed in *Cucumis sativus* (28) and *Cucumis melo* (29).

The results obtained with the multiplication medium $M_{m2,}$ compared with the induction medium M_{i2} (without BAP) confirm once again that BAP is a crucial factor for the adventitious shoot regeneration of *Cucurbitaceae* family. Indeed the lowest number of shoots per explant among the induction media was observed on M_{i2}, without BAP. Among the multiplication media, M_{m2} (differing from M_{i2} by the presence of BAP and absence of IBA, GA_3 and Kinetin) increased the number of shoots per explant, with 8.6 ± 2.5 shoots (Table 2). This value was similar to the number of shoots obtained on the best multiplication medium, i.e. M_{m3}. Compton *et al.* (9) obtained the same results. Their work revealed that the presence of 1 mg/l BAP in the culture medium facilitated shoot multiplication.

The results obtained with the rooting medium M_{r2} were similar to those obtained with watermelon (*Citrullus lanatus*) by Compton *et al.* (9) who noted that shoots of at least 15 mm length, rooted easily. The plantlets

from medium M_{r2} with a length higher than 15 mm acclimatized easily and showed highest percentage of acclimatization. Theses results were in accordance with the study of Compton *et al*. (9, 10), indicating a correlation between plantlet length and acclimatization survival.

Conclusion

The study showed that indigenous oilseed *Citrullus lanatus* can be efficiently propagated via organogenesis using cotyledon proximal part with hypocotyl segment explants excised from 5-day-old seedlings and single node explants from 20-day-old seedlings. That opens a way to cucurbit genetic improvement for increasing the yield potential of this crop and contributing significantly to food security and poverty alleviation. However, the protocol needs to be improved in order to increase the number of shoots induced per explant and to reduce the time necessary to produce seedlings ready to be acclimatized. To address this problem, parameters such as the amount of mineral elements, growth regulators, organic compounds and amount of agar will have to be optimized. Moreover, the plants obtained *in vitro* should be evaluated morphologically in greenhouse or in the fields to assess the genetic integrity of the regenerated plants.

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