AcademicPres

Available online at www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not. Bot. Hort. Agrobot. Cluj 38 (2) 2010, Special Issue, 141-146

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

In Vitro Preservation of Yam (*Dioscorea cayenensis – D. rotundata* complex) for a Better Use of Genetic Resources

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Abstract

Among the food crops, yam takes up quantitatively the first place in the gabonese diet. Unfortunately, it can stay available only 6 to 7 months in the year because of difficulties of harvest and post- harvest. This problem is little studied in the case of *Dioscorea cayenensis-D. rotundata* complex. In order to optimize the use of micro tubers for the growing in green house or field, it is important to control the duration of storage before the germination. The present study concerns microtubers obtained by *in vitro* culture. When microtubers were harvested (after 9 months of culture) and directly transferred on a new medium without hormone, the tubers rapidly sprouted in *in vitro* conditions. Harvested microtubers were also stored dry in jars in sterile conditions during 2 to 18 weeks before *in vitro* sprouting. In this case, microtubers stored during 18 weeks sprouted more rapidly than those stored 8 weeks. The size of the tubers used for the storage had great influence on further sprouting. The upper microtubers in 25 mm can be kept to the darkness, under 50% of relative humidity, in 25°C during at least 18 weeks. Sprouting is 100% whatever the substrate of culture. The plant tissue culture technique constitutes a serious alternative for the preservation of plant kinds and for the production of planting material. These techniques allow multiplying in a short time of thousands of copies of new varieties of newly created plants. These *in vitro* plants can be used on one hand, for the production planting material, and on the other hand for ex vitro storage of breeding grounds with decelerated growth, to struggle against genetic erosion. These results should allow improving in practice the multiplication of yam, while guaranteeing phytosanitary qualities

***K*eyswords:** yam, storage, sprouting, *Dioscorea cayenensis - D. rotundata,* microtubers

Introduction

The yam (*Dioscorea cayenensis- D. rotundata* complex) is an important food plant in many tropical countries in Asia, South America and Africa. It is the leading food crop in Gabon in terms of tonnage produced which contributes about 24% of the human energy (Coursey, 1983). This nutritional function can only be fulfilled if availability is guaranted by suitable storage systems. The vegetative cycle of the yam only allows one harvest per year, and the early cultivars can be eaten about 4 months before the later cultivars. Therefore, to ensure year-round availability, a storage period of 8 months is needed. The yam, like other root and tuber crops such as cassava and taro, suffers post harvest losses ranging from 25 to 60% (Degras *et al*., 1977). These storage losses are caused partly by external agents, such as insects, rodents and moulds. The high water content of the tubers combined with damage occurring during or after harvesting, makes them vulnerable to micro-organisms. In the yam tuber, sprout initials are formed beneath the periderm just prior to breakage of dormancy (Onwueme, 1973). The sprouting process accelerates the losses and limits storage life, starts with the appearance of the sprouting loci. The production of yams is seasonal, so storage is necessary before planting and for use as food. Harvesting is done 180 days after planting (Ile *et al*., 2006). After harvest, tubers enter into dormancy, which is of major importance in yam storage. Once sprouting occurs, storage is no longer possible. Even though the yam is cheaper and of superior quality than other products, the production is hindered by storage problems (Treche *et al*., 1996), Jaleel *et al*., 2007).

Hence, long dormancy of tubers is a desirable attribute in yam breeding and selection programs (Shiwachi *et al*., 2003). It permits a better storage but it complicates the use of the tuber for propagation, however. Irrespective of when a yam seed tuber is planted, the critical starting point of the growing season is when dormancy ends sprouts are produced. There is a period of about four months after harvest during which tuber losses are incurred in storage but propagation of the planting material would not be successful. In some environments, early planting could largely obviate losses incurred during seed-yam storage if they were more flexibility in the control of sprouting date through effective means of artificially terminating dormancy. Many workers used plant growth regulators to break or to prolong dormancy but results are inconsistent (Barker *et al*., 1999).

The aim of the present study was to determine good conditions for storage of yam microtubers and further

sprouting. These conditions can be helpful for the use of microtubers from selected yams as "seeds" by the growers.

To optimize the use of microtubers for the culture in greenhouse or in the field by the farmers, it is important to master the parameters such as the duration of storage and the delay for the further sprouting. The present work describes the incidence of the storage duration, temperature and the size of the microtubers on sprouting delay and sprouting rate. This study should help the development of a method for a rapid mass production, storage and sprouting of microtubers of the *Dioscorea cayenensis-D. rotundata* complex

Materials and methods

In vitro cultures of *D. cayenensis-D. rotundata* complex was provided by the Roots and Tubers Transit Centre of CIRAD (France). The identification number were clones CTRT 233 and CTRT 234 both coming from two different tubers of cv. 'Singo'. Axillary shoot proliferation was maintained by subculturing single nodes, every 2 months on MS salt medium (Murashige and Skoog,1962) supplemented with vitamins of Morel (Morel *et al*., 1951) and containing 30 gl^{-1} sucrose, 2 gl^{-1} activated charcoal and 8.2 gl^{-1} Caldic agar (Hemiksem, Belgium). The pH of the media was adjusted to 5.7±0.1 before autoclaving at 121°C for 20 min. Cultures were maintained in a 16-h photoperiod (Sylvania Grolux fluorescent lamps, 50 $\mu molm^2s^{-1}$) at a day/night temperature of 25/22°C.

For tuberisation, cuttings (2 cm long) with one leaf were cultured in glass containers (800 ml) with plastic lid containing 125 ml of the same medium (Ondo Ovono *et al*., 2007). The tubers were harvested for this study after 9 months. Microtubers obtained were then used for storage and sprouting experiments.

For storage, harvested microtubers were kept in darkness, in 800 ml closed glass jars without medium, five per jar. All treatments consisted of two replicates (separated by one month) with 20 tubers in each. Different storage conditions were tested on the further sprouting of yam *Dioscorea cayenenesis –D. rotundata* microtubers.

Day temperature: generally 25°C but compared with 18°C in one experiment (Fig. 1)

Relative humidity. In normal conditions, when tubers were put directly in glass jars, the humidity was around 47%. In some experiments, two layers of filter paper (9 cm diameter) moistened with 3 ml distilled water were laid down in the jars before the tubers. In this case, the relative humidity in the jars was close to 100%.

Tuber size. Generally, only tubers with a length superior of 2.5 cm were used. In some experiments (indicated on figures), microtubers were distributed according to the size in three categories: Small, Medium and Large based on the length. The tubers failing in between 1.5-2 cm were considered as small, 2.5-3 cm as medium-sized and above 3.5 cm as large. The tubers below 1.5 cm were rejected.

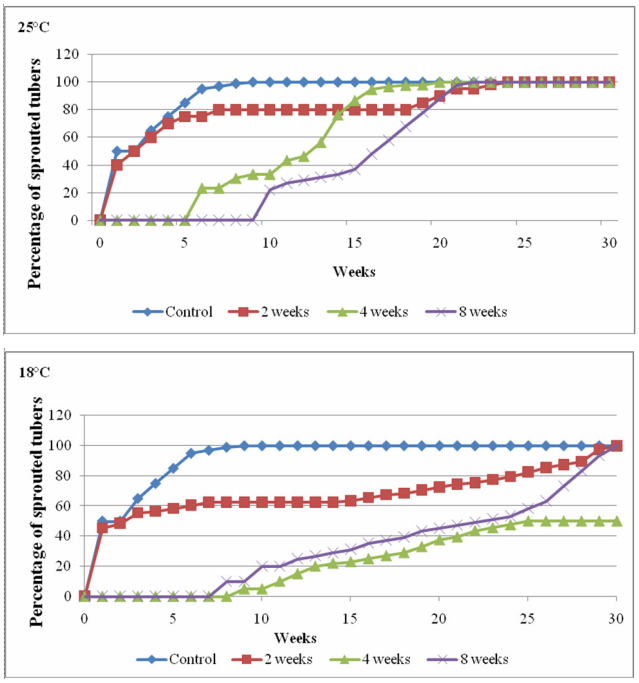

Fig. 1. Effect of storage duration in darkness (0 to 8 weeks) at two temperatures (25 or 18°C) on sprouting of yam microtubers (clone CTRT 233). Control = without storage

Sterility. Generally, the glass jars used for storage were previously sterilized and the manipulations were done under sterile airflow. In one case (Fig. 2), the jars were not sterilized and the manipulations were done directly in the lab.

Duration: from 2 to 18 weeks.

Directly (control) or after several weeks of storage, tubers were transferred on MS medium without hormones for sprouting in the conditions used for proliferation. In some cases, sprouting was compared in sterile and non-sterile composts in greenhouse under relative humidity close to 100% during the two first weeks. The temperature of the greenhouse was 26°C the day and 22°C at night.

Sprouts occurred at the head portion of *Dioscorea* tubers around the point of detachment from the mother plant. Sprouting rates were observed every 7 days. When one bud reached a length of 2 mm, tuber was considered as sprouted.

Data represents means of 20 microtubers/treatment, repeated two times.

Results and discussion

To use *in vitro* yam microtubers as seeds, it is important to be sure that all the tubers are able of sprouting. *In vitro* production can be done all over the year but the seeding has to take into account seasonal changes. Meanwhile, microtubers must be stored. But, as for traditional tuber cultures, microtubers could present a dormant period. The mechanism of dormancy in yam tuber, and for that

matter in other tuberous species too is poorly understood and so the physiological dormancy period cannot be easily predicted or manipulated (Orkwor *et al*., 1998). Long dormancy of tubers is a desirable attribute in yam breeding and selection programs. It permits a better storage but it complicates the use of the tuber for propagation.

When microtubers were harvested and directly transferred on a new medium without hormones, the tubers rapidly sprouted. After one week, around 50% of the tubers have sprouted and after 8 weeks, all the tubers have sprouted (Fig. 1, 25°C). Dormancy in traditional culture is widely assumed to start at or shortly after tuber maturity and most studies begin measuring dormancy time from harvest. However various studies showed that in fact field tubers are dormant well before harvest (Craufurd *et al*., 2001; Wickam *et al.,*1984). In the case of the present microtubers harvested after 9 months of *in vitro* culture, no dormancy was observed. In these conditions, is it possible to store the microtubers?

Various factors were studied concerning their storage and their further sprouting:

- Storage duration. The first question was the influence of the storage duration on the further sprouting. The microtubers used in our experiments did not present dormancy if they are transferred *in vitro* on a new medium directly after harvest. On the contrary, if the microtubers were stored at 25°C, the sprouting was delayed. The longer was the storage time, up to 8 weeks, the longer was the delay (Fig. 1). In the case of 8 weeks storage, a delay of 9 weeks was needed before observing the first sprouting and 22 weeks were needed to observe 100% of sprouting. The delay of sprouting was quite similar after 12 weeks of storage (Fig. 2a). In this case, no sprouting was observed before 10 weeks and 18 weeks were needed to obtain the sprouting of all the tubers. When the storage was longer, the delay of sprouting decreased and after 18 weeks of storage, the sprouting was very rapid. Around 35% sprouting was observed after 2 weeks and after 8 weeks, all the microtubers have sprouted (Fig. 2a). It seems thus that storage induced a "dormancy-like period" and this dormancy was maximum between 8 and 12 weeks of storage, i.e. the start of sprouting and the time needed to observe 100% sprouting were maximum. After 18 weeks of storage, the results were reversed, almost similar to the control without storage, only one week of delay. A "dormancy-like" state was induced progressively between 2 and 8 weeks of storage (Fig. 1). Its duration (storage time + sprouting delay) was between 20 weeks for the first tubers to sprout and 28 weeks for the slower. A so "fixed" dormant period (culture time + delay of sprouting) was already observed by Okali (1980) in traditional yam culture. Further, it will be interesting to check if a longer storage is possible.

The storage temperature has a significant effect on dormancy and the delay of sprouting in traditional culture (Mozie,1988). The reduction of the storage temperature for microtubers from 25°C to 18°C induced an increase of the delay needed to observe the sprouting of all the tubers (Fig. 1). More than 24 weeks were needed when the tubers were stored 2 or 4 weeks at 18°C while all the tubers

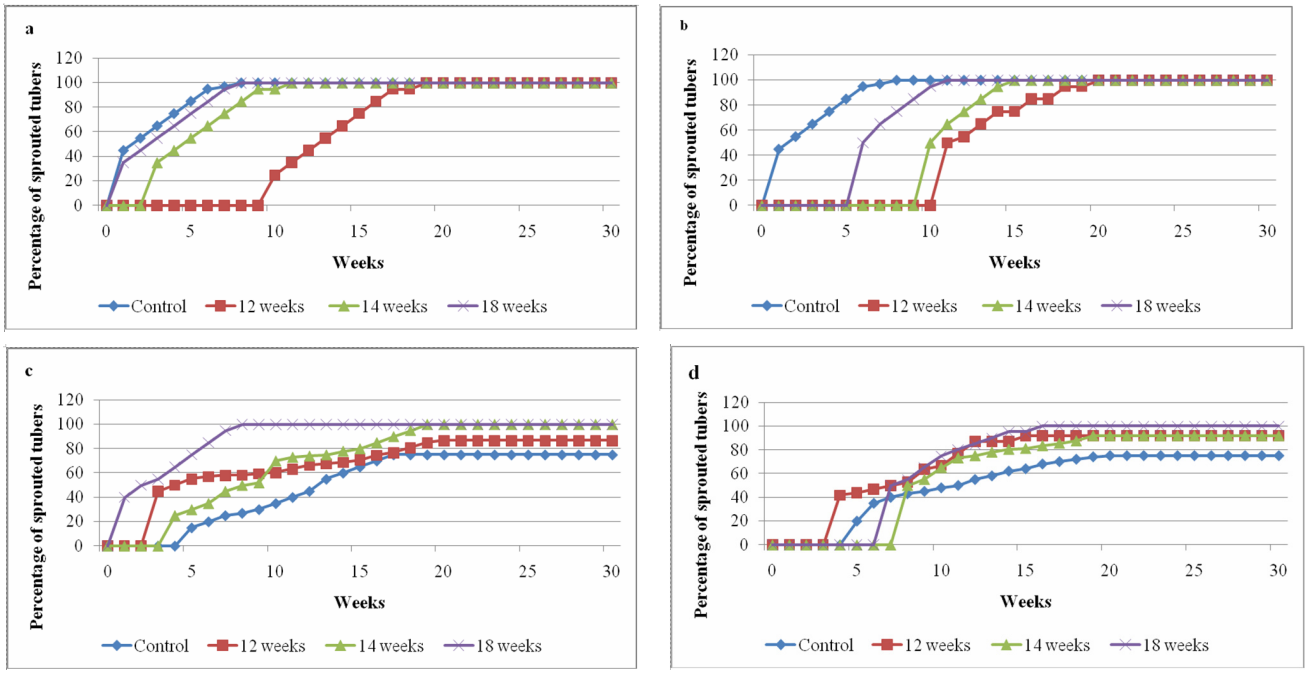

Fig. 2. Effect of storage duration (0 to 18 weeks), in sterile and non sterile conditions on sprouting of yam microtubers (clone CTRT 233) at 25°C in darkness; a: in sterile jars, *in vitro* sprouting; b: in non-sterile jars, *in vitro* sprouting; c: in sterile jars, sprouting in sterile compost; d: in sterile jars, sprouting in non-sterile compost

stored at 25°C showed sprouting after 20 weeks. After 8 weeks of storage at 18°C, the difference was still more important, only 50% of tubers sprouted after 28 weeks. Thus, the delay for sprouting of the microtubers would be longer with the decrease of the storage temperature. These results showing an increase of the "dormancy like period" with a decrease of temperature were in agreement with those obtained by Mozie(1987) in traditional culture. Tubers stored at 16°C remained dormant for between 120 and 150 days longer than those stored at 21 to 32°C. For *in vitro* potato tubers, similar effect of lowering results with storage temperature were observed and a role for endogenous growth regulators as cis-zeatin and cis-zeatin riboside (Suttle, 2000), starch and amylose contents (Leeman *et al*., 2005; Kaur *et al*., 2007) was shown in the regulation of dormancy. Decreasing storage temperature could be a means to increase yam microtuber dormancy and so to increase storage time. However these tubers showed a more heterogenous sprouting, which is not a desirable characteristic for field cultivation. The temperature used in the following experiments was 25°C.

The sterility during the storage conditions had also an influence on the sprouting rate (Fig. 2). Compared to sterile conditions, storage in non sterile conditions for 14 or 18 weeks further delayed sprouting. It is possible that the development of pathogens can interfere with the "dormancy-like" breaking process. The sterility of the storage conditions could ensure a better reproducibility of the results and was used in all following experiments.

The relative humidity. Two different relative humidities in the closed jars used for tuber storage were tested on their further sprouting. In the previous experiments, the humidity in the jars varied between 45% and 50% during the storage. We tested a relative humidity higher than 95% during the storage. No significant difference was found on the sprouting rate after storage in the two conditions (data not shown). This result is in agreement with the observations of Akoroda (1995) on tubers from traditional culture. The process of drying and hardening the surface skin of tuber prior to storage did not have any effect on duration to sprouting. On the contrary, in *D. spicufolia*, storing tuber segments (from traditional culture) at 32°C and with a low relative humidity delayed sprouting relative to tubers stored at the same temperature with high relative humidity (Preston and Haun, 1963). This difference can be related to the cutting of the tubers of *D. spicufolia* and hence a difference in the loss of moisture for the tuber.

The microtuber size. Already in 1988, Alsadon *et al.* (1988) have shown that larger microtubers of potato were able to produce a larger crop than small ones when they were transplanted or directly sown into the field. In yam, the effect of microtuber size before storage was also very important for the further sprouting (Fig. 3). The sprouting of large microtubers (length above 3.5 cm) occurred earlier than that of the smaller tubers (length between 1.5 to 2 cm) sprouted (Fig. 3a). In addition, final sprouting rate was only 40% for the smaller tubers. Quality of seed potato depends on the starch content, which is related to

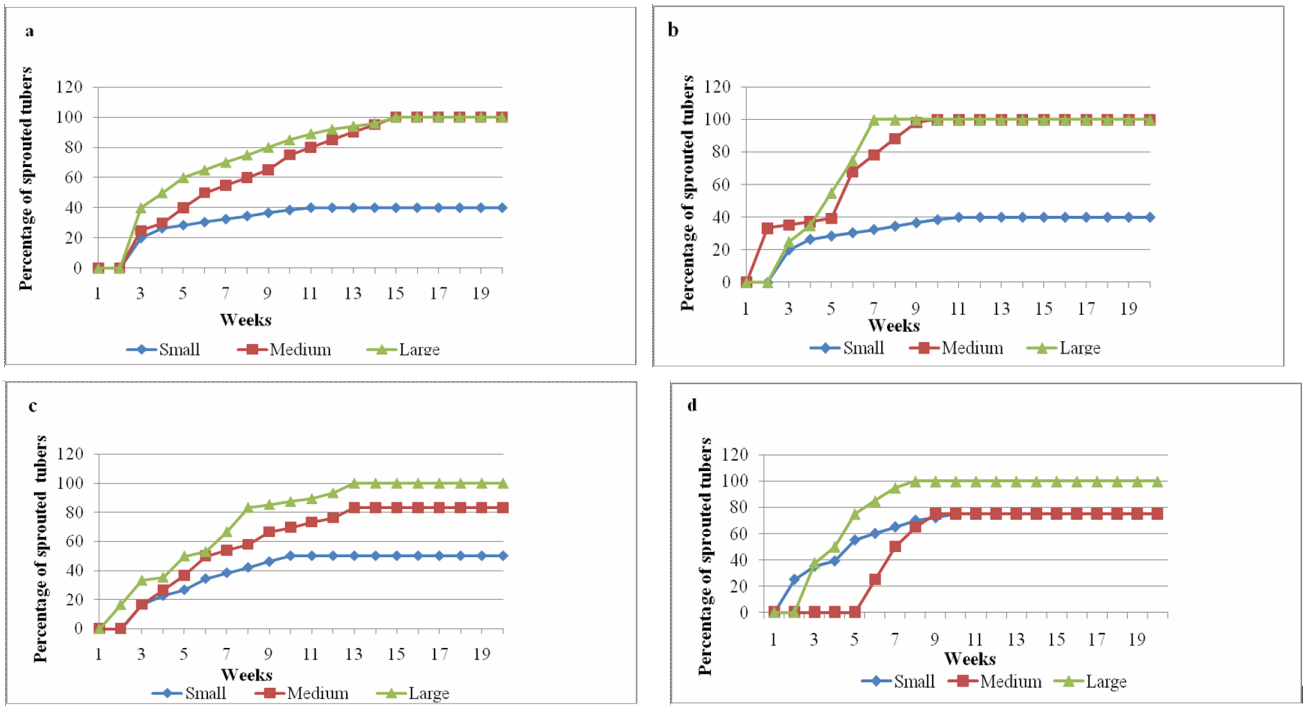

Fig. 3. Sprouting after storage (25°C, 14 weeks) in darkness or light conditions, of different size classes of microtubers obtained on different tuberisation media (Yam clone CTRT 234); a) tuberisation on free hormone medium, storage in darkness; b) tuberisation on free hormone medium, storage in light (photoperiod 16h); c) tuberisation on medium supplemented with 10µM J, storage in darkness; d) tuberisation on medium supplemented with 10µM JA, storage in light (photoperiod 16h)

sprouting vigor (Park *et al*., 2001). Bigger microtubers had more reserves and thus could more easily sprout although the dormant period was generally associated with a minimum of endogenous metabolic activity, resulting in very little loss of storage reserve. These results also confirmed those of (Vakis,1986) who showed that more field tubers were physiologically old (=large) more the seeding was fast. When microtubers were physiologically young (=small), sprouting was slower. Then, by aging, their germinal vigor increased and, consequently, the seeding was accelerated. Nevertheless storage in darkness was a better solution because it resulted in a more homogenous 100% sprouting, for the medium-sized and the large tubers. These storage conditions are more practical for farmers. The smallest tubers should not be used.

Ex vitro sprouting. To have practical implications, it was necessary to transfer this technique to normal soil. After storage, the microtubers were transferred in the greenhouse for sprouting (Fig. 2c, d). Freshly harvested microtubers began to sprout after 4 weeks when they were transferred in sterile or non sterile compost while *in vitro* the first sprouting was observed after one week (Fig. 2 a). Moreover in compost, only 75% of these microtubers sprouted. After 12 weeks of storage, the sprouting was earlier in sterile or non sterile compost than in *in vitro* conditions. The "dormancy-like" state observed after 12 weeks storage in *in vitro* conditions was broken more rapidly in compost. After 18 weeks storage, the sprouting in sterile compost was similar to that observed in *in vitro* conditions: around 40% sprouting after one week and 100% after 8 weeks. In non sterile compost, the delay was more important but 100% sprouting was also observed. Thus, *ex vitro* sprouting was possible without special difficulties.

Conclusion

In these studies, interesting results were obtained when microtubers were harvested after 9 months of culture and were kept dry in jars in sterile conditions during 2 to 18 weeks before sprouting in *in vitro* conditions. The size of the tubers used for the storage had great importance for further sprouting. A storage temperature of 25°C permit a quicker sprouting than 18°C. *Ex vitro* sprouting was not a problem. The delay for sprouting was more important in contrast to *in vitro* conditions but a rate of 100% was reached. This fact was very important for an agronomical application of this technique for the production of selected "seeds" (microtubers).

Acknowledgments

Dr Filloux from CIRAD for providing the material, GABON government for awarding scholarship to PO and the skilful assistance of the APE personnel (provided to CEDEVIT by the government of Wallonia).

This work was already published: P. Ondo Ovono, C. Kevers, J. Dommes: Effects of storage conditions on sprouting of microtubers of yam (*Dioscorea cayenensis-D. rotundata complex*). Comptes Rendus, Biologie, 2010, 233:28-34.

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