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Influence of various carbohydrates on the *in vitro* micropropagation of *Nauclea diderrichii* (De Wild &T. Durand) Merrill, an endangered forest species in Togo

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The over exploitation of *Nauclea diderrichii*, for its very resistant wood against the attacks of fungus (*Coriolus versicolor*), Lyctus, termites (*Reticulitermes santonensis*) and marine borers, leads to its disappearance in Togo. This forest species produces many fruits containing numerous seeds; unfortunately their seedling is laborious in its biotope. *In vitro* micropropagation trials were carried out for a faster and massive regeneration. The effect of carbon source on the rooting and the growth of seedlings were studied in the presence of sucrose, glucose, fructose, mannitol, maltose, galactose, mannose, lactose, sorbose and sorbitol. Woody plant medium (WPM) of Lloyd and McCown has been used. This medium was modified by supply of microelements and vitamins of Murashige and Skoog and supplemented by 30 g/L of these carbohydrates. The best plants' growth and rooting were obtained with sucrose. Sucrose appears to be the most favorable sugar to ensure the *in vitro* micropropagation of *N. diderrichii*. Sorbose caused the necrosis of explants in culture. The mannose and the mannitol, did not cause the necrosis of explants, but their presence had an unfavorable role on roots initiation and slow down the plants' growth.

Key words: Sources of carbon, *Nauclea diderrichii*, *in vitro* micropropagation, Togo.

INTRODUCTION

The composition of the culture medium determines the *in vitro* growth of plants. Sugar in culture medium has been considered the sole carbon source for the growth of cells, buds, shoots, and even plantlets (Gauchan, 2012). The

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main components of most plant tissue culture media are mineral salts and sugar as carbon source and water (Gamborg and Phillips, 1995). Sugar is a very important component in medium and its addition is essential for *in vitro* growth and development of plants because photosynthesis is insufficient, due to the weak development of their leaves, the limited gas exchange and the high relative humidity (Pierik, 1987; Kozai, 1991; Mazinga et al., 2014). Organized tissues show a better growth and proliferation after the addition in medium an adequate source of carbon (Mazinga et al., 2014). Sugars enter the metabolism pathways and transformation of energy which are required for growth of cell (Gauchan, 2012). Except sucrose which is more often used in tissue culture, others carbohydrates are successfully used as a source of carbon. However, the same carbohydrate can give very controversial effect according to the species. The fructose gave good results for culture of *Morus alba* (Oka and Ohyama, 1986), *Castanea sativa* (Chauvin and Salesses, 1988) and *Malus pumila* (Welander et al., 1989), but it gave bad results in *Malus* Jork (Moncousin et al., 1992), *Syringa chinensis* (Welander et al., 1989) and *Prunus cerasus* (Borkowska and Szczerba, 1991). As for the glucose, it showed successful results in *Alnus* (Welander et al., 1989), *Potentilla fruticosa* and *Ficus lyrata* (Wainwright and Scrace, 1989). The effect of carbohydrates on the micropropagation can differ according to the species (Baskaran and Jayabalan, 2005). We therefore performed *in vitro* tests to study the effects of diverse sources of carbon in *N. diderrichii* during the present survey. *N. diderrichii* is a forest species belonging to the family of Rubiaceae. Its regeneration *in situ* is laborious because of the dormancy of its seeds (Hawthorne, 1995a). Despite the abundance of fruits produced by trees every year, the regeneration by seed is very low. Under the mother-trees or far from them, no future stem had been identified on the field (Adjonou et al., 2014). In Togo, the overexploitation and lack of this species reforestation leads to its disappearance, as a result this species is classifies on list of the rare species (UNEP, 2010).

The *in situ* deficit of seedling is very worrisome when we know that the species is overexploited for its very resistant wood against the attacks of fungus (*Coriolus versicolor*), Lyctus, termites (*Reticulitermes santonensis*) and marine borers by local sawyers (Gérard et al., 1998; Soro et al., 2014). Furthermore, some of its organs (barks) are used in traditional medicines (Dibong et al., 2013). Its *in vitro* regeneration can be a good alternative to supply a stock of seedling of plantation (Pitekelabou et al., 2015). The study carried out here, is consisted to determine the efficiency of various sources of carbon on the rooting and the shoots development of seedlings, in order to identify the source of carbon adapted for *in vitro* micropropagation of *N. diderrichii.*

MATERIALS AND METHODS

Vegetal material used was constituted by uninodal fragment of seedlings stemming from *in vitro N. diderrichii*'s seeds germination. Culture is initiated on Woody plant medium (WPM) composed by Lloyd's and McCown's WPM macroelements (1980), 100 mg/L of myo-inositol, Murashiges' and Skoog's, microelements and vitamins (1962). This basic medium was alternately supplemented by each of the following carbohydrates: sucrose, maltose, lactose, glucose, fructose, galactose, sorbose, mannose, mannitol and sorbitol at 30 g/L rate. The ten culture media, differing by the source of carbohydrates, were solidified with agar-agar at 8 g/L. Their pH has been adjusted between 5.6 and 5.7 with NaOH at 1 N or HCl at 1 N.

These media were then distributed in tubes of 20 × 150 mm and sterilized at 120°C in autoclave under 1 bar during 20 min. Two months old *in vitro* plants were cut in uninodal fragments then put in culture on these ten media. Three months later, uninodal fragments cultivated in the presence of mannose or of mannitol, were transferred on a medium containing 30 g/L of sucrose. Tubes containing the *N. diderrichii's* explants are stored in a culture room with a photoperiod of 16 h at 27 ± 2°C and a light intensity of 120 $\mu Em^{-2}s^{-1}$. The light was supplied by fluorescent lamps.

Observations were performed during six weeks. At the end of every week, the number of roots, shoots, and nodes was noted and the shoots and roots length are measured. Every measure was made on a population of 20 individuals and has been repeated twice. The results presented in this work correspond to those obtained after six weeks of culture. Data were subjected to the variance analysis (one-way ANOVA) and means were classified in homogenous groups according to Newman and Keuls's range test ($\alpha = 0.05$) using Statistica version 10 (Statsoft Inc; Tulsa, USA: 2011) program.

RESULTS

Various carbohydrates used during this work gave diverse results. Sorbose showed a very noxious effect on the *N. diderrichii's* explants. Indeed none of them survived after 2 weeks of culture. Hundred per cent of necrosis was observed (Table 1). On galactose medium, 65% of explants sprouted, and no late evolution appeared except signs of necrosis. The bottom of explants took an orange color and leaves presented a typical yellowish aspect of senescence (Fiure 1). The use of sorbose or galactose caused sooner or later total necrosis of plants. On the other hand the use of mannose or mannitol has allowed 70% of explants to sprout but no late development of these sprouted explants was able to be observed (Figure 1).

Sucrose in culture medium allowed the best growth of roots which length (3.05^{d} ± 1.01 cm) is significantly different from that of plants' roots cultured in presence of all others carbohydrates used in present study (Table 2). It was noticed that in presence of sucrose, the shoots were bigger with longer internodes (Figure 1). Other carbohydrates: maltose, lactose, glucose, sorbitol and fructose, allowed the seedlings to produce roots, but did not favor a good growth of these. This low growth of the roots with length which was not more than 1.91^{a} ± 0.70 cm

Table 1. Effect of various carbohydrates on the viability and shoots development of *N. diderrichii*'s explants.

Sugar (30 g/L)	Necrosis explants (%)	No sprouted explants (%)	sprouted explants without growth
Saccharose	0[a]	0[a]	0[a]
Maltose	0[a]	0[a]	0[a]
Lactose	0[a]	0[a]	0[a]
Fructose	0[a]	0[a]	0[a]
Sorbose	100[c]	0[a]	0[a]
Glucose	0[a]	0[a]	0[a]
Galactose	25[b]	10[a]	65[b]
Mannose	0[a]	30[b]	70[b]
Sorbitol	0[a]	0[a]	0[a]
Mannitol	0[a]	0[a]	100[c]

Average ± standard deviation of the measures made on 20 explants and repeated twice. The values affected by the same letter in the same column, are not significantly different according to the test of Newman-Keuls in $P < 0.05$.

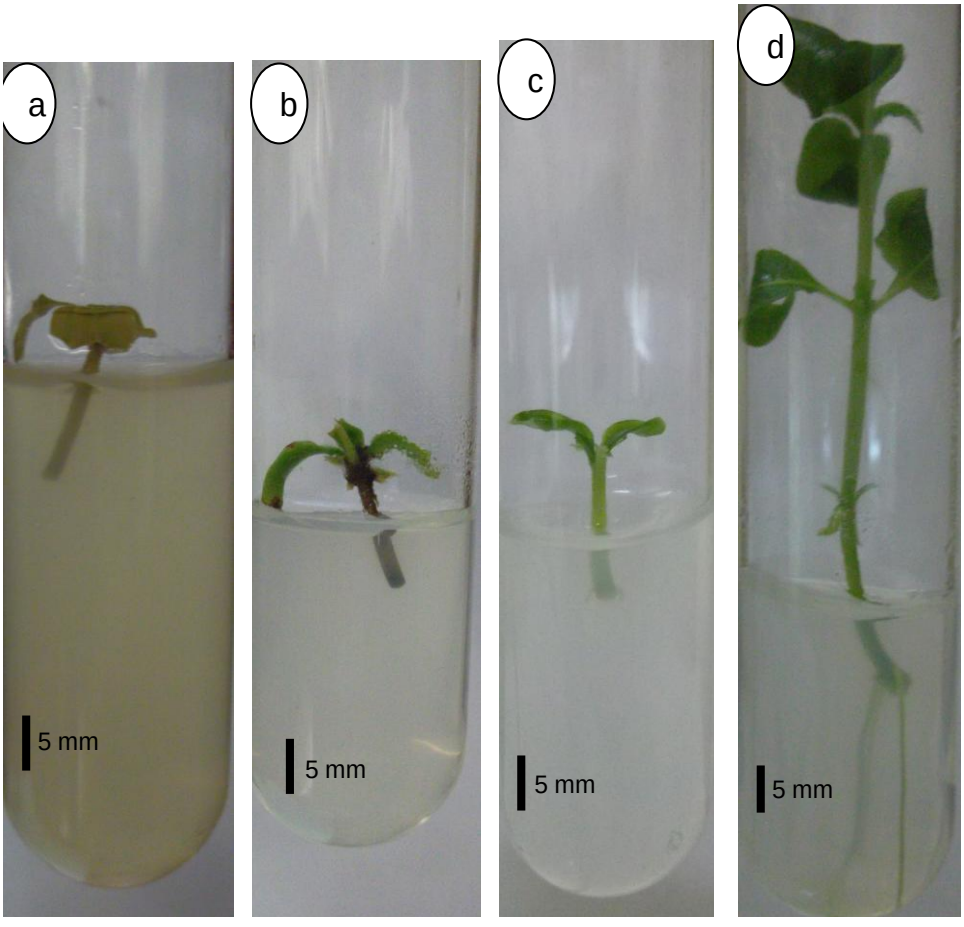

Figure 1. Development of seedlings on WPM medium containing different sugars. **(a)** Necrosis plant on sorbose. **(b)** Progressive necrosis plant on galactose. **(c)** Sprouted plant without growth on mannito. **(d)** Normal growth of plant on sucrose.

Table 2. Effect of various carbohydrates on rooting of the explants of *N. diderrichii.*

Carbohydrate (30 g/L)	Number of Roots / plant	Length of roots (cm)
Saccharose	$3.35^{a} \pm 1.18$	$3.05^{d} \pm 1.01$
Maltose	$3.20^{a} \pm 1.36$	$1.91^{a} \pm 0.70$
Lactose	$3.40^{a} \pm 1.19$	$1.41^{a} \pm 0.66$
Fructose	$3.80^{a} \pm 1.40$	$0.69^{c} \pm 0.73$
Sorbose	-	-
Glucose	$2.95^{ac} \pm 1.32$	$1.87^{a} \pm 1.01$
Galactose	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
Mannose	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
Sorbitol	$2.35^{c} \pm 1.23$	$1.45^{a} \pm 0.66$
Mannitol	$0.50^{b} \pm 0.89$	$0.03^{b} \pm 0.05$

Average ± standard deviation of the measures made on 20 explants and repeated twice. The values affected by the same letter in the same column, are not significantly different according to the test of Newman-Keuls in $P < 0.05$.

Table 3. Effect of various carbohydrates on the development and the growth of the explants of *N. diderrichii.*

Carbohydrates (30g/L)	Nodes/plant	shoots/plant	Length of plant (cm)
Saccharose	$4.00^{c} \pm 1.56$	$2.30^{d} \pm 1.08$	$3.96^{c} \pm 1.71$
Maltose	$2.60^{e} \pm 0.94$	$1.25^{a} \pm 0.44$	$2.43^{b} \pm 0.84$
Lactose	$2,00^{d} \pm 0.79$	$1.15^{a} \pm 0.37$	$1.80^{b} \pm 0.75$
Fructose	$4.00^{c} \pm 0.86$	$1.90^{c} \pm 0.72$	$2.42^{b} \pm 0.85$
Sorbose	-	-	-
Glucose	$3.25^{f} \pm 0.97$	$1.55^{ac} \pm 0.60$	$2.18^{b} \pm 0.77$
Galactose	$0.65^{a} \pm 0.49$	$0.65^{b} \pm 0.49$	$0.07^{a} \pm 0.05$
Mannose	$0.70^{a} \pm 0.47$	$0.70^{b} \pm 0.47$	$0.07^{a} \pm 0.05$
Sorbitol	$1.45^{b} \pm 0.51$	$1.10^{a} \pm 0.31$	$0.60^{a} \pm 0.34$
Mannitol	$1.20^{ab} \pm 0.41$	$1.20^{a} \pm 0.41$	$0.10^{a} \pm 0.00$

Average ± standard deviation of the measures made on 20 explants and repeated twice. The values affected by the same letter in the same column, are not significantly different according to the test of Newman-Keuls in $P < 0.05$.

was even lower in fructose presence. Galactose or mannose in the medium inhibits completely rooting of *N. diderrichii's* plantlets and the addition of mannitol led to low rhizogenic plantlets ($0.50^{b} \pm 0.89$ root/plant) with null virtually growth ($0.03^{b} \pm 0.05$ cm) of shoots (Table 2).

Sucrose in culture medium allowed moreover the best growth of shoots which length ($3.96^{c} \pm 1.71$ cm) was significantly different from that of shoots cultured in presence of all others carbohydrates used. Indeed the length of plants obtained on medium supplemented with maltose, lactose, glucose, or fructose was not more than $2.43^{b} \pm 0.84$ cm when on medium supplemented with sorbitol, mannose, mannitol or galactose the plants' growth was virtually null ($\leq 0.60^{a} \pm 0.34$ cm) (Table 3). From all the sugars used in this work, sucrose gave greater shoots number ($2.30^{d} \pm 1.08$ shoots / plant). The greater multiplication rate which was 4.00 nodes / plant was obtained in this study in presence of sucrose or fructose, but the internodes of shoots obtained on fructose medium were very short and all of these nodes could not be recuperated for future multiplications.

Explants stemming from mannitol or mannose medium and transferred on medium of sucrose at 30 g/L gave the results carried in Table 4. The length of seedlings stemming from the medium containing sucrose is significantly different from the seedlings stemming from the medium containing mannose or mannitol. On the other hand, no significant difference was noted between the stemming

Table 4. Development of explants stemming from the first put in culture of six weeks in the presence of sucrose, mannose and mannitol and transferred on a medium containing sucrose.

Origin of explants	Roots/plant	Length of roots (cm)	Nodes/plant	Shoots/plant	Length of plant (cm)
Stemming from Sucrose	$3.35^{a} \pm 1.18$	$3.05^{a} \pm 1.01$	$4.00^{a} \pm 1.56$	$2.30^{a} \pm 1.08$	$3.96^{b} \pm 1.71$
Stemming from mannose	$1.35^{b} \pm 0.81$	$2.93^{a} \pm 1.43$	$4.45^{a} \pm 2.48$	$3.00^{a} \pm 1.84$	$2.81^{a} \pm 1.41$
Stemming from mannitol	$2.80^{a} \pm 0.95$	$2.98^{a} \pm 1.11$	$5.70^{a} \pm 3.34$	$3.55^{a} \pm 2.24$	$2.95^{a} \pm 1.21$

Average ± standard deviation of the measures made on 20 explants and repeated twice. The values affected by the same letter in the same column, are not significantly different according to the test of Newman-Keuls in $P < 0.05$.

seedlings of sucrose, mannose or mannitol concerning the average number of shoots, nodes and the roots length averages by plant. The multiplication rate was statistically the same for three types of origin of explants (Table 4).

DISCUSSION

The internal carbohydrate pool is suggested to have an important role in organogenesis of several woody species (Kromer and Gamian, 2000). However the exogenous supply of carbon sources can influence this organogenesis (De Neto and Otoni, 2003). The carbon sources serve as energy and osmotic agents to support the growth of plant tissues (Lipavska and Konradova, 2004). In present study, sucrose, maltose, lactose, fructose, glucose and sorbitol allowed all the root initiation of *N. diderrichii's* plantlets. However, according to these different sugars above, a significant difference was noticed on the growth of these different roots. Indeed, the length of initiated roots on the medium containing sucrose was significantly different from those obtained with others sugars used in this study. Sucrose, not only has been very favorable to the roots growth, but it has also favored the better growth of plantlets. The similar results were obtained for *Centell asiatica* L. (Anwar et al., 2005), *Pogostemon cablin* Berth (Swamy et al., 2010), *Solanum nigrum* Linn (Sridhar and Naidu, 2011). On the contrary, Preethi et al. (2011) observed that the fructose gave better results than sucrose, maltose and glucose used for the micropropagation of *Stevia rebaudiana*. For *Rosa rugosa*, Xing et al. (2010) showed that the action of the glucose was more favorable than sucrose for the proliferation and the growth of shoots and then the presence of sucrose in medium led to a yellowing of the leaves of plantlets. For peach rootstock GF 677 (Ahmad et al., 2007) and *Prunus armeniaca* (Marino et al., 1991), it was rather sorbitol which favored better efficient rooting and growth of plantlets than sucrose. On the other hand, Rahman et al. (2010) noted no significant difference when using sucrose, maltose and glucose in the *in vitro* culture of five varieties of potatoes. No sign of chlorosis was observed in the presence of sucrose in this work, while it provoked the chlorosis and the progressive death of shoots for *Prunus Mume* (Hisashi and Yasuhiro, 1996) and *Eclipta alba* (Baskaran and Jayabalan 2005). The growth and root initiation are highly energy requiring processes that can occur at the expense of available metabolic substrates, which are mainly carbohydrates (De Klerk and Calamar, 2002). Generally, sucrose is the carbohydrate used most of the time in the culture medium of tissues cultivated *in vitro* (Sul and Korban, 2004; Fuentes et al., 2000), because of the facilitated absorption of sucrose through the cellular membrane (Borkowska and Szezebra, 1991). In this work, among all sugars used, sucrose has been the one which has allowed obtaining not only a good rooting, but also a better growth of roots and plantlets. That suggests that the *N. diderrichii*'s plantlets absorb better sucrose and benefit from this sugar better energy than others sugars used here. The better growth of plantlets obtained in this work under effect of sucrose can be understand also in fact that this sugar in favoring the better growth of roots, favors the better absorption of nutritive substances in culture medium by plantlets. This reason has allowed to obtain the better shoots/plant and a good multiplication rate on this medium. According to Cuenca and Vieitez (2000), the ability to metabolize different types of carbohydrates differs within the plant kingdom. So the negative effects of some sugars on the plant growth are caused by their inefficiency metabolization by cells of these crops or by their reduced uptake in these plant species (Jain et al., 1997). These could be the main causes of a low growth of roots and plantlets of *N. diderrichii* on some media in this study. Galactose, mannose or mannitol in the culture medium inhibited the roots initiation partially or completely. The growth of plantlets was null in presence of these three carbohydrates. In galactose presence the plantlets finally necrotized. Similar result was found by Arditti and Ernst (1984) at orchids. In presence of mannose or mannitol, no plant was affected by necrosis, or developed any deformity. Sorbose has provoked total necrosis of all the explants of *N. diderrichii*. The result of these four types of sugar suggests that these sugars absorbed little or not and so provide few energy to *N. diderrichii* plantlets preventing them to initiate roots and have a good

development. However plants cultivated in the presence of the mannose or mannitol and transferred later on a medium containing sucrose developed well. No significant difference was noted between the rooting, the multiplication rate of these plants and those of the native plants of the medium containing sucrose. So the mannose or mannitol in the culture medium by slowing down the growth of *N. diderrichii*'s plants, can serve to keep this species' *in vitro* plants and a fast and massive multiplication can be done at the moment needed by transfer on the medium containing sucrose. A similar result was reported in the presence of mannitol for *Vanilla planifolia* where this carbohydrate slowed down the growth and allowed to keep this plant *in vitro* during seven years by making only a subculture / year (Divakaran et al., 2006). Charoensub and Phansiri (2004) also showed that the mannitol reduces the growth and the number of shoots by plant for *P. indica* Linn. According to the work of Da Silva and Scherwinski-Pereira (2011) with *Piper aduncum* and *P. hispidinervum*, the addition of the mannitol (1-3%) in the culture medium reduced the growth of seedlings, but with much higher contents, it causes the necrosis of seedlings. However the lethal concentration is dependent on the botanical species.

Conclusion

In the present study, observations showed that, the rooting and multiplication of shoots of *N. diderrichiis* seedlings *in vitro* are affected by the type of exogenous carbon source added to the medium. Among the different carbon sources used in this study, sucrose has been proved to be better for shoots' proliferation, growth and rooting of *N. diderrichii*'s seedlings, than other carbon sources in micropropagation. The divers effects obtained according to the carbohydrate in the culture medium confirm that a plant answers differently according to the carbohydrate as reported by other authors. It is thus imperative to adapt the protocol of micropropagation to every species by determining, in particular, the type of optimal carbohydrate to the development of the *in vitro* Plants' cultures. Besides, mannose and mannitol added in low concentration, in the culture medium slowed down and even stop the *N. diderrichii's* seedling growth without any later damage for these. The stabilizing action of these two carbohydrates on this botanical species' growth would allow to store and keep at a lower cost *in vitro* plants of *N. diderrichii*.

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