Effect of Nitrogen Nutrition on the *in vitro* Tuberization of *Xanthosoma sagittifolium* (L.) Schott (Cocoyam)

J.J.T. TSAFACK[AB], T. BOUDJEKO[A]., D.H. MBOUOBDA[A]., D.N. OMOKOLO[A]

[a]High Teacher's Training College, University of Yaounde I P.O. Box 47, Yaounde, Cameroon

[b]Institute of Medical Research and Medicinal Plant Studies P.O. Box 6163, Yaounde, Cameroon

ABSTRACT

The influence of nitrogen status in the culture medium on microtuberization of *Xanthosoma sagittifolium* (L.) Schott, cv white was studied on Murashige and Skoog (1962) medium free-growth regulators bud supplemented with variable NO_3^-/NH_4^+ ratios. Statistical analysis showed that microtuberization of cocoyam is correlated positively with NO_3^-/NH_4^+ ratio. The rate of tuberization increased with the ratio. Similarly, the ability of microtubers to develop buds increased with NO_3^-/NH_4^+ ratio. Infact, with 4:1 ratio, 60 % of formed microtubers developed buds. In our experimental conditions, two distinct phases of tuberization process were distinguished: the initiation phase and the filling phase. A high increase in soluble sugars preceded the initiation phase. During the filling phase, the sugar content in the microtubers was highest in media with 2:1 and 4:1 ratios. Thin layer chromatography revealed that these sugars could be mainly glucose, fructose and sucrose.

Key words: Nitrogen, *in vitro* tuberization, *Xanthosoma sagittifolium* (L.) Schott

Corresponding author: domokolo@uycdc.uninet.cm

INTRODUCTION

Tuberization is a complex developmental process, which depends on the interaction between genotypic and environnemental factors. Among the environmental factors, photoperiod, thermoperiod, sugars, phytohormones are documented determinants (Kefi *et al.*, 2000; Omokolo *et al.*, 2003). Nitrogen nutrition also constitutes a key factor in this process. The level and source of nitrogen influence the potato (*Solanum tuberosum* L) microtuberization (Garner and Blake, 1989). Chen and Liao (1993) found that the fresh weight of the potato tubers decreased as a result of decreasing nitrate/ammonium ratio without changing total nitrogen concentration. High levels of nitrogen are known to be inhibitory of tuberization through reduction of the carbohydrates/nitrogen ratio. Furthermore soluble carbohydrates may act as substrate that triggers tuberization, as energy source as osmoticum preventing growth. They are also important regulators of gene expression (Ewing and Struik, 1992).

Cocoyam (*Xanthosoma sagittifolium* L. Schott) is a tuber food crop of the family of Araceae, attaining world importance as energy food. It provide carbohydrates and essential amounts of protein, fat and vitamins. In Cameroon, cocoyam is second among the root and tubers crops (Onokpise *et al.*, 1999). With the opening of new areas of consumption, cocoyam has gained economic importance in tropics and subtropics countries (Giacometti and Léon, 1994). However, vegetatively propagated commercial varieties are prone to pathogens, thereby resulting in very poor quality and yield due largely to a root rot disease principally caused by *Pythium myriotilum* (Nzietchueng, 1985). The principal vector of dissemination of pathogens is the corm fragment, which serve traditionally as planting material. Consequently, much attention has been focused on the efficient *in vitro* production of disease-free seed cocoyam plantlets (Omokolo *et al.*, 1995). Another approach to improve cocoyam via micropropagation is the *in vitro* production of microtubers. The use of microtubers can offer several advantages: they are convenient for handling, storage and transport of germplasm. Moreover, they can be used as experimental research tools for biochemical, physiological and genetic studies of the plant (Coleman *et al.*, 2001). For these reasons, efforts have been made to increase microtuberization of cocoyam while a reduction in the complexity of media is also desirable, but no information is available on the associated biochemical changes.

The present study is a contribution to the search for optimum conditions of microtuberization process in cocoyam and its associated biochemical changes. The aim is to examine the influence of nitrogen nutrition -precisely the effects of NO_3^-/NH_4^+ ratio- on *in vitro* tuberization and on soluble carbohydrates accumulation during this phenomenon in *Xanthosoma sagittifolium*.

MATERIALS AND METHODS

Plant material The plant material was made up of cocoyam vitroplants (cv white) of 2-3 cm height, with 2-3 leaves. Those explants were obtained following the modified micropropagation technique of Omokolo *et al.* (1995).

Culture media

The basal medium used was the Murashige and Skoog (1962) mineral salts medium supplemented with Morel and Wetmore vitamins, (1951), 4 % (w/v) Agar and 3 % (w/v) sucrose. Micropropagation was carried out on the basal medium enriched with 10^{-5} M Benzylaminopurin (BAP).

Tuberization was induced on the basal medium supplemented with 8 % (w/v) sucrose, NO_3^- and NH_4^+in the following ratios 1:4, 1:2, 1:1, 2:1, 4:1.

Culture conditions

Micropropagation of the shoot tip of cocoyam was done for 40 days under a photoperiod of 16:8 (70 µmol m^{-2} s^{-1}) at 25±1°C. For microtuberization, a photoperiodic regime in short days (SD: 8h light) for 10 days was applied with a thermoperiod of 25/20 ± 1°C (SD^t_{10}) followed by a transfer of cultures to total darkness for 50 days (SD^t_{10}-$dark_{50}$). The microtuberization was realised in an incubator (incubator Selecta HotCold-GL, model EC500 GL, Spain) with relative humidity 75 ± 4 % and irradiance of 70µmol m^{-2} s^{-1}.

Sampling and Extraction

The basal parts of the explants were collected at days 5 and 10, then with an interval of 10 days until 60 days. The collected sample was washed, allowed to drain; weighed and then stored at –20°C for biochemical analyses. Two grams of samples (fresh weight) were ground in a mortar and boiled under reflux in 6 ml of 80 % (v/v) ethanol for 20 min (two changes). The ethanol-soluble extracts were filtered through filter paper (Whatman n°3) and concentrated under vacuum at 50°C prior to analyses.

Table 1: Microtuberization response in cocoyam as influenced by ratio NO_3^-/NH_4^+

Parameters	$NO3^- / NH4^+$ 1:4	1:2	1:1	2:1	4:1
Tuberization (%)	23.33	28.00	41.33	36.33	27.33
Budding (%)	43.66	48.66	65.33	49.33	60.00
Mean number of microtubers /explant	1.66(1.33)	2.33(1.33)	3.33(2.33)	3.66(2,66)	2.33(0.66)
Mean weight of microtubers/explant (g)	0.89 ± 0.14^{b}	0.90 ± 0.20^{b}	1.80 ± 0.30^{a}	$1.29\pm0,36^{a}$	0.95 ± 0.12^{b}
Mean diameter of microtubers (mm)	3.26 ± 0.10^{ab}	3.60 ± 0.24^{b}	4.03 ± 0.18^{a}	5.83 ± 0.19^{c}	3.63 ± 0.11^{b}

Means having different letters are significantly different according to HSD- Tukey test at $P\leq0.05$

Determination of total and reducing carbohydrates

Soluble carbohydrates were determined using the Anthron method of Ashwell (1957) and the reducing sugars by the method of Somogyi (1952).

Identification of individual sugars was made by Thin Layer Chromatography using methanol: ethyl acetate:acetic acid: water (1:6:1.5:1) as migrating solvent. Sugars were revealed by spraying the plates with a solution containing 2 ml of 50 % (w/v) diphenylamine in aniline, 100 ml acetone and 15 ml phosphoric acid. The spot were developed after steming plates at 110 °C for 10 min. Sugars were identified by comparing Rf of the sample with Rf of pure standard analytical grades of sucrose glucose and fructose.

Data analysis

Forty eight explants were used per experiment and all experiments were repeated three times. After 60 days, the percentage of explants undergoing tuberization and budding was estimated. For each experimental condition, the average number, weight and diameter of microtubers were also estimated. Triplicate samples were taken for each experiment and analysed for total soluble and reducing sugars. The data were processed with Principal Component analysis (PCA) using the "SPAD, release 3.5" and ANOVA using Tukey-HSD multiple range tests of the "SPSS statistical package, release 8.0" for windows.

RESULTS

Effects of NO_3^-/NH_4^+ ratio on the microtuberization

The anatomic differentiations, a direct consequence of the tuberization process begins to appear 5 to 10 days after induction. The immature microtubers obtained after 60 days of culture on different nitrogen nutrition conditions were all yellowish, spherical and smooth (Fig. 1A). Two months later, mature microtubers were rough and covered with brown epidermic extensions (Fig. 1B).

Generally the rate of tuberization was low in

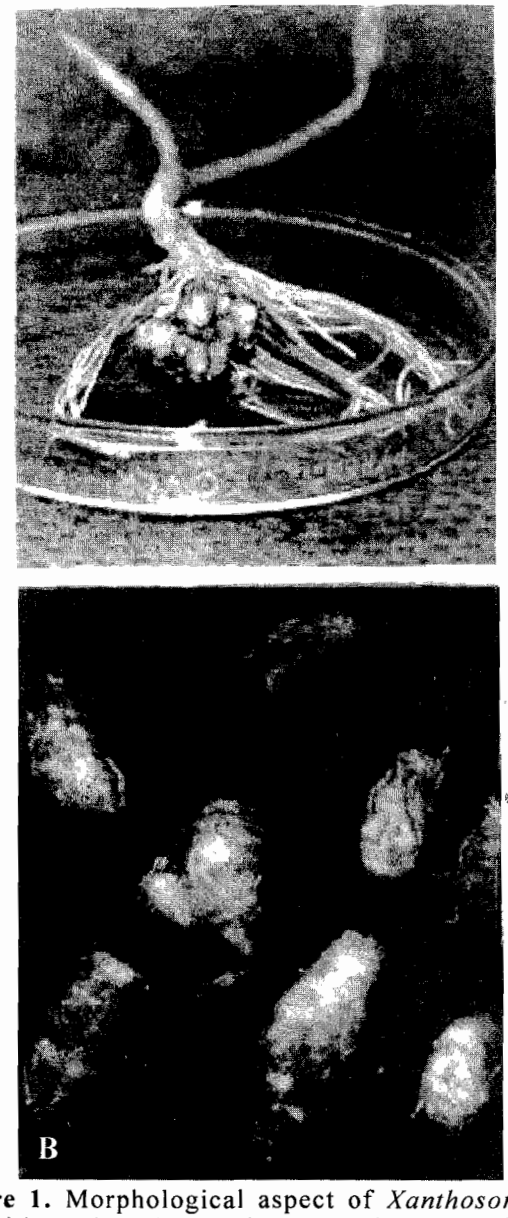

Figure 1. Morphological aspect of *Xanthosoma sagittifolium* microtubers; A: immature microtubers after two months; B: mature microtubers after 4 months. (bar = 1.5 cm)

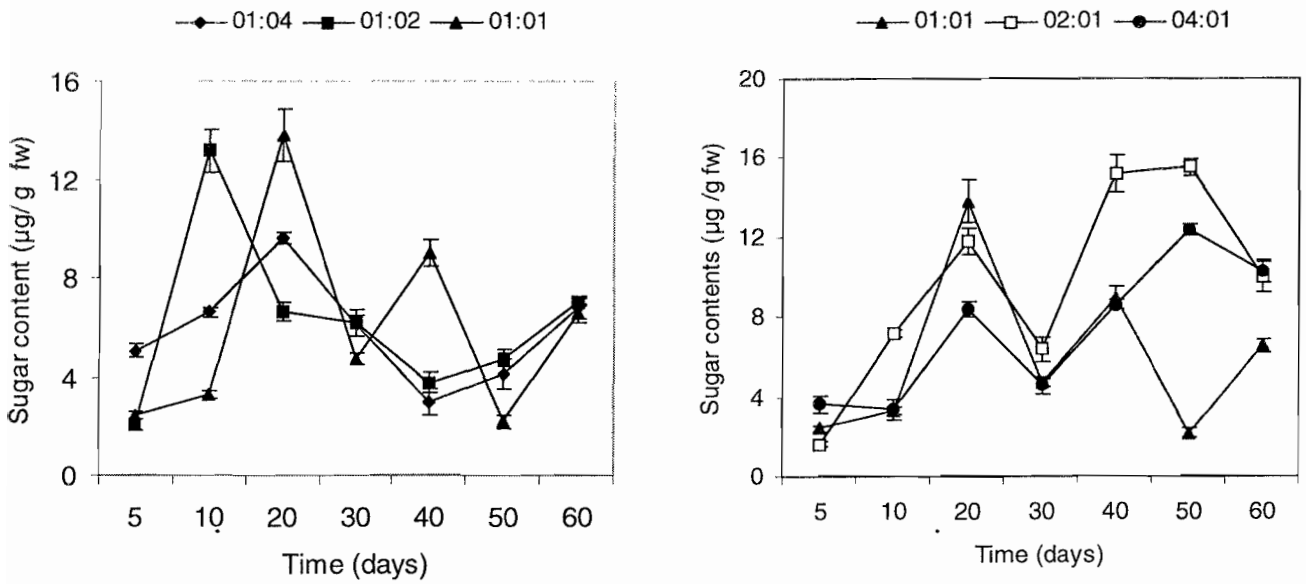

Figure 2: Changes in total soluble sugars content of *Xanthosoma sagittifolium* microtubers induced in media with various NO_3^-/NH_4^+ ratios A: with NO_3^- dominant; B- with NH_4^+ dominant

growth regulator free medium. When the basal medium was supplemented with 1:4, the rate of tuberization was lowest (22.33 %). However it progressively increased with NO3-/NH4+ ratio up to 1:1. From there, it gradually decreased until 27 % when NO3-/NH4+ ratio reached 4:1 (Table 1). The ability of microtubers to develop buds increased with NO3-/NH4+ ratio. Infact, the budding rate increased from 43.66 % when NO3-/NH4+ ratio was 1:4, to 60 % with 4:1 ratio (Table 1).

A mean of 2.66 principal and 1.66 secondary microtubers were recorded per explants. The number of principal microtubers increased from 1.66 when NO3-/NH4+ ratio was lowest (1:4) to 3.66 when it was highest (2:1). Above 2:1 ratio, it decreased sharply until it rose to 2.33 when the NO3-/NH4+ ratio reached 4:1. The mean number of secondary microtubers followed the same pattern. The mean weight of microtubers per explants was highest (1.80 g) at 1:1 ratio. It was oscillating 0.90 g with 1:4, 1:2 and 4:1 ratios. The mean diameter was 3.26 mm at 1:4 ratio. Thereafter it gradually increased up to 5.83 mm when the ratio was 2:1. Above this ratio, the mean diameter decreased until it rose up to 3.63 mm with 4:1.

Effects of NO_3^- / NH_4^+ ratio on carbohydrates content

The evolution of total soluble sugar contents presents two lines of increase, interrupted by 2 peaks located between 10th or 20th days and 40th or 50th days of culture in respective of NO_3^-/NH_4^+ ratio.

The beginning of tuberization was characterized by an increase in total soluble sugar content with

Table 2: The total soluble (A) and reducing sugars contents correlation matrices (B)

Table 3: Relative amounts of sugars as estimated by TCL of microtubers soluble carbohydrates at 30 and 60days after the *in vitro* tuberization induction.

Time (days)	Sugars	NO_3^- / NH_4^+				
		1:4	1:2	1:1	2:1	4:1
30	Glu	+	+	++	+	+
	Fruc	-	-	+	++	+
	Suc	+	++	++	+	++
60	Gluc	++	+	+++	++	+
	Fruc	-	-	+	+	-
	Suc	+	+	++	++	++

+++: Very high intensity; ++:high intensity; +:low intensity; -absent Gluc: glucose; Fruc:fructose; Suc:sucrose

a peak observed at day 10 for 1:2 ratio and day 20 for the 1:4, 1:1, 2:1 and 4:1 ratios (Fig. 2A and 2B). The highest soluble sugar contents (13.80 and 6.64 µg/g fresh weight) were registered at day 20 with 1:1 and 1:2 ratios respectively (Fig. 2A). After day 20, it significantly decreased with the NO_3^-/NH_4^+ ratio, but it doubled with 2:1 and 4:1 ratios where NO_3^- was dominant (Fig. 2B). The PCA test showed that there was a positive correlation between NO_3^-/NH_4^+ ratios 4:1 and 2:1 (r_p=0.84, P<0.05); 1:4 and 1:2 (r_p=0.46, P<0.05) (Table 2). The accumulation of soluble sugars takes longer time when NO_3^-/NH_4^+ratios were 1:4 and 1:2.

The variation of reducing soluble sugar content followed the same pattern. At day 5, 68.60 % of sugars were reducing soluble sugars. The reducing soluble sugar content increased with time and reached its highest peak 20 days after induction of microtubers, giving the sugar content of 9.30, 9.50, 9.66, 7.60 µg/g fw respectively for 1:4, 1:1, 2:1 and 1:4 ratios (Fig. 3A and 3B). A peak was reached at day 10 (10.22µg/g fw) with 1:2 ratio (Fig. 3A). A second peak was observed at day 50 when NO_3^-/NH_4^+ratios were 1:4, 1:1 and 2:1. It was recorded least, after 40 days of culture when the ratio was maintained at 4:1 (Fig. 3B). Generally, at the beginning of microtuberization the difference between reducing sugars content recorded at different NO_3^-/NH_4^+ ratios was not statistically significant (P<0.05). However, after the transfer of explants to darkness (at the end of culture), the quantity of reducing soluble sugars was reduced to the half with 1:4 and 1:2 ratio. This was 2.5 times less than that of ratios favourable to NO_3^- ions. When comparing the reducing soluble sugars contents, the positive correlations were recorded between 2:1and 4:1 (r_p=0.71, P<0.05); 2:1 and 1:1 (r_p=0.54, P<0.05) (Table 2). The PCA

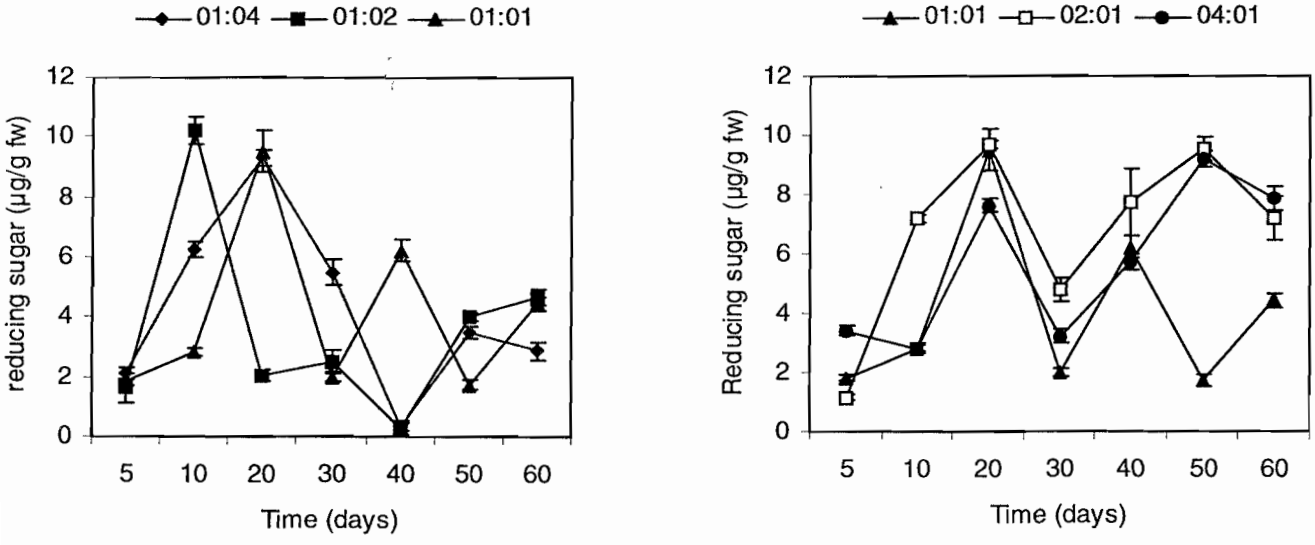

Figure 3. Changes in reducing soluble sugars content of *Xanthosoma sagittifolium* microtubers induced in media with various NO_3^-/NH_4^+ ratios A: with NO_3^- dominant; B- with NH_4^+

test showed that the quantity of reducing soluble sugars required for microtuberization was relatively synthesized during a short period time with $NO3^-$/ $NH4^+$ ratio favourable to nitrate.

Sugar identification

Thin layer chromatography (TLC) profiles of ethanolic extracts of soluble carbohydrates revealed that glucose and sucrose were present in the cocoyam microtubers (Table 3). 30 days after induction, the presence of 3 sugars (glucose, fructose and sucrose) was revealed with ratios 1:1, 2:1and 4:1; 2 sugars (glucose, sucrose) with ratios of 1:2 and 1:4. After 60 days of induction, a general increase in the intensity of spots was observed except when NO_3^-/NH_4^+ ratio was 1:4.

DISCUSSION

Results showed that the morphogenetic and biochemical parameters associated with microtuberization could be influenced by the nitrogen status of the culture medium. Generally the tuberization rate was low (31.06 %) on the growth regulator–free media when compare with the results reported by Omokolo *et al.* (2003) with MS basal medium supplemented with BAP. This rate varies with NO_3^-/NH_4^+ ratio. Microtuberization process was improved when nitrate was higher or equal to ammonium. This suggests that the NO_3^- ion like in potato (*Solanum tuberosum* L.) have favourable effects on *in vitro* tuberization in cocoyam (Garner and Blake, 1989). Nitrate is the most oxidized mineral form of nitrogen. It is easily soluble in aqueous solution and is therefore more absorbable by plants. It facilitates the cellular uptake of cations (Heller *et al.*, 1996). The ability of microtubers to develop buds increases with NO_3^-/NH_4^+ ratio; with 4:1 ratio, 60 % of formed microtubers developed buds. However the mean rate of budding generally remains very high.

The differentiation of microtubers into buds shows that there could be an interaction between growth and tuberization. Vecchio and Pagano (1996) showed that in potato low level of nitrogen creates endogenous conditions which favour microtuberization. Nitrogen is an essential growth element: it favours vegetative growth and increases the intensity of green colour of leaves. The budding of microtubers could also be attributed to the enrichment of the basal medium. In potato, the contact of microtubers with the Murashige and Skoog (1962) basal medium induces some endogenous factors that favours budding (Charles *et al.*, 1995). The inhibition of budding, the vegetative dormancy and the induction of tuberization could constitute a successive chain of the same physiological process (Rousselle *et al.*, 1996). The nitrogen source also has an effect on the development of microtubers. The diameter, the number and the weight of microtubers equally show that the inhibitory effect of nitrogen in cocoyam may be essentially due to $NH4^+$ ions. Similar results were recorded with higher nitrate content. In this case, the inhibition is due to the loss of equilibrium between the growth of the aerial part of the plant and that of the microtubers (Rousselle *et al.*, 1996). These results fully agree with the work of Garner and Blake (1989) who demonstrated that nitrogen nutrition in *S. tuberosum* was a limiting factor to the height of microtubers.

The evolution of total soluble and reducing carbohydrates content permitted to detect the two tuberization phases as defined by Sihachakr *et al.* (1982) on *Ipomea batatas*: the initiation phase and the filling phase. These phases are also encountered in other tubers crops such as *Dioscorea alata* and *Solanum tuberosum* (Akogo *et al.*, 1995; Charles *et al.* 1995). In cocoyam, the initiation phase is preceded by a rapid increase in sugar content which disappears 5 to 10 days later. The evolution of sugar content during initiation phase has been noted by Mangat *et al.* (1990). They reported that in *Begonia rex*, the initiation of organogenesis was characterized by an accumulation followed by a rapid disappearance of carbohydrates. Similarly, Naidu and Kishor (1995) and Coleman *et al.* (2001) found that the reducing sugar content was high in developing tubers. These sugars play an important role during tuberization. They are indispensable for the formation of storage polysaccharides such as starch. TLC profiles revealed that glucose and fructose may be the principal reducing sugars. They might result from the enzymatic hydrolysis of sucrose, a channel of reduced carbon. The reducing sugars actively help to increase the biomass of tubers as well as their enrichment in amylaceous reserves (Coleman *et al.*, 2001). Rousselle *et al.* (1996) showed in their work on *Solanum tuberosum* that nitrogen is one of the key component of the culture medium known for its effect on the tuberization process. The mechanism of its action is attributed to $NH4^+$ ions.

CONCLUSION

The aim of this work was to study the effects of the nitrogen source on the *in vitro* tuberization of cocoyam. Our results show that:

-the NO_3^-/NH_4^+ ratio influence the ability of cocoyam to develop tubers, buds and to accumulate

soluble sugars;

-a nitrate concentration higher or equal to that of ammonium (2:1 and 1:1 ratios) in the culture medium improves the microtuberization process in cocoyam;

-2 distinct phases of microtuberization were characterised: the initiation phase and the filling phase,

-the initiation phase is preceded by an increased in soluble sugars, which are mainly glucose, fructose and sucrose.

Further research is required on the nitrogen-induced biochemical changes to refine this conclusion.

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