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African Journal of Biotechnology Vol. 6 (7), pp. 923-928, 2 April 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Optimum conditions for cotton nitrate reductase extraction and activity measurement

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Accepted 17 June, 2006

Conditions of nitrate reductase extraction and activity measurement should be adapted to plant species, and to the organs of the same plant, because of extreme weaknesses and instabilities of the enzyme. Different extraction and reaction media have been compared in order to define the best conditions for cotton callus nitrate reductase activity measurement. The use of potassium phosphate buffer, at pH 7.5 allowed for a better stabilization of the enzyme during extraction. The addition of 15 mM of glutamine in the extraction buffer stimulates significantly, *in vitro*, the reduction of nitrate. Enzyme activity is moreover optimal when 1 M of exogenous nitrate, as substrate, is added to the reaction medium. At these optimum conditions of nitrate reductase activity determination, the substrate was completely reduced after 20 min of enzyme incubation, and the greatest velocity of nitrate transformation into nitrite (µg of NO_2^-/min/g F.W) is observed when incubation period of enzyme is short (1 to 5 min).

Key words: Extraction, dosage, nitrate reductase activity, callus, cotton.

INTRODUCTION

Nitrate reductase (EC. 1.7.99.4) is an oxidoreductase enzyme involved in nitrogen assimilation in plant. It intervenes in the reduction of mineral nitrogen (NO_3^-) to nitrite (NO_2^-), and to ammonia ($NH4^+$) for protein synthesis (Nakagawa et al., 1985; Rajeseckhar and Oemuller, 1987). The reduction is accomplished by two subunits of the enzyme which use molybdenum and iron as cofactors (Buc et al., 1995).

Nitrate reductase is very weak and instable during extraction (Campbell and Smarrelli, 1986; Mori, 2000). Extraction and dosage of the enzyme must be adapted to plant species, even to different organs of the same plant, to get a better enzyme response (Rajeseckhar and Oemuller, 1987; Campbell, 1999). The extraction of the enzyme is generally done with different buffer solutions, containing protecting agents according to organs or plant species. Curtis and Smarrelli (1986) used a potassium phosphate buffer for the extraction of nitrate reductase from soybean. Kuo et al. (1982) used a Tris buffer for barley nitrate reductase extraction and Aslam and Oaks (1975) employed HEPES buffer for corn nitrate reductase. Berrehar (1988) and Robin (1979) demonstrated the essential rule of polyvinylpirrolidone (PVP), antioxidant substances and cysteine in the extraction medium, in order to obtain a better stability of the enzyme.

Optimum conditions for cotton nitrate reductase extraction and activity measurement are yet to be established. In this work, optimum conditions of callus nitrate reductase extraction and activity in two local cotton cultivars (Kouakou et al., 2004) were examined. Proteins involvement in somatic embryogenesis has been

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Abbreviations: TRIS, tris (hydroxylmethyl) amino methane; PVP, polyvinylpirrolidone; HEPES, N-2 hydroxylethylpiperasine-N-2 ethanesulfonic acid; PEG, Polyethyleneglycol; FW, fresh weight..

established by different authors (Trolinder and Goodin, 1988; Kwa et al., 1995). Nitrate reductase is known to play a key role in the control of ammonia content in tissue and cells, and to promote somatic embryogenesis of many plant species (Bayley and Gamborg, 1972; Kouakou et al., 2004). The best buffer to use for cotton nitrate reductase extraction, aminino acid effect on enzyme extraction and the effect of substrate concentration in the reaction medium, were discussed in relation to the optimum response of the enzyme.

MATERIALS AND METHODS

Plant material

Cotton callus (*Gossypium hirsutum* L.), cultivar ISA 205 N from Centre National de Recherche Agronomique (CNRA), Côte d'Ivoire, was used for the experiments. Callus was initiated from 1 cm hypocotyls of 5 days-old plantlets. *In vitro* seed germination, explants culture for callus development has already been described (Kouadio et al., 2004).

Extraction and nitrate reductase activity measurement

Nitrate reductase activity was investigated based on the method used by Nakagawa et al. (1985). A 0.5 g callus was ground in 5 ml cold buffer containing 0.05 g of PVP; 0.5% of polyethylene glycol (PEG); 0.25% of sodium thiosulphate and 15% of glycerol. The extract was filtered using a 25 ml of sephadex G25. Nitrate reductase activity determination was based on the formation of nitrite in a reaction medium containing 0.1 ml of enzyme extract, 0.2 ml of NADH (13 mM) and 1.2 ml of buffer. The reaction was terminated after a certain period of incubation by adding 1 ml of nathtylethylene diamine – HCl (0.02%) and 1 ml of sulfanilamide (1%) to the medium. Enzyme activity was expressed in µg of nitrite (NO_2^-) produced/min/g of callus fresh weight (F.W.).

Optimum conditions of enzyme extraction and activity

The selection of the best conditions for enzyme extraction and activity measurement was achieved by modifying the Nakagawa method (Nakagawa et al., 1985). To find out the best extraction buffer of cotton nitrate reductase, enzyme was extracted in the following buffer at pH 7.5; potassium phosphate, 0.1 M, Tris-HCl, 0.1 M, HEPES, 0.1 M, sodium borate, 0.1 M and sodium acetate, 0.1 M.

To select the best pH buffer, enzyme was extracted with the best buffer used in the previous study, at different pH values of: 5, 5.5, 6, 6.5, 7, 7.5, 8, 9 and 10.

Different concentrations of cysteine and glutamine (0, 1, 5, 10, 15, 20 and 25 mM) were added individually in the optimum extraction buffer at the optimum pH, to see the effect of amino-acids on the stability of the enzyme.

The concentration of substrate which gives the best enzyme response is determined by using different KNO_3 concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5 and 1 M) in the reaction medium.

Enzyme activity kinetic

Kinetic of nitrite formation by nitrate reductase is followed by the enzyme activity measurement, after period of incubation of 1, 5, 10, 20, 25, 30 and 60 min when enzyme is extracted and its activity measured under optimal conditions.

Statistical analysis

Data are analyzed using ANOVA/MANOVA, with mean comparisons by Newman-Keuls test at α =5%.

RESULTS AND DISCUSSION

Composition of the best extraction buffer of cotton nitrate reductase

Variable activity of cotton nitrate reductase enzyme was obtained with the 5 buffers used in this work (Table 2). Three classes of buffer were observed. The first was represented by potassium phosphate buffer which yielded the highest enzyme activity (2.82 µg of NO_2^-/min/g F.W.). The second class was represented by Tris-HCl and HEPES buffers. Theses buffers exhibited the similar enzyme activities (1.8 and 1.97 µg of NO_2^-/min/g/ F.W.). The third class was represented by sodium borate and sodium acetate buffers which resulted in lower enzyme activities (0.96 and 1. 07 µg of NO_2^-/min/g F.W.). The optimum activity of nitrate reductase is dependent upon extraction and activity measurement conditions. Thus, the extracting buffer is carefully chosen, according to its chemical composition, capacity to resist the presence of acidic and alkaline substances, cellular membrane permeability and pH stability to the variation of temperature (Boyer, 1986). Nitrate reductase is a very weak and unstable enzyme during the extraction. Its activity is variable from one plant species to another (Campbell, 1999). These results show an important variability of cotton callus nitrate reductase response, depending on the composition of extraction buffer.

Phosphate buffer is the best buffer for cotton nitrate reductase extraction. This buffer is generally used for the extraction of many plant species nitrate reductase (Nakagawa et al., 1985; Curtis and Smarrelli, 1986). However, Aslam and Oaks (1975) showed that the best activity of corn nitrate reductase is obtained when this enzyme is extracted with HEPES buffer. Phosphate buffer is physiologically inactive and does not react with bivalent ions as Mg^2+ and Ca^2+. Moreover, it inhibits only the enzymes which use phosphate as reacting substances (Boyer, 1986). That did not seem to be the case for cotton callus nitrate reductase.

Tris and HEPES buffer gave similar enzyme activities, which is lower than the activity of the enzyme when it is extracted in phosphate buffer. These two buffers had in common the presence of alcohol (ethanol and methanol) and acids (HCl and H_2SO_4) in their chemical composition (Dawson et al., 1986). These substances seem to inhibit the reaction of reduction of nitrate in cotton callus. These buffers can also react with metallic ions (Constans, 2004). The inhibitory effect of Tris and HEPES might due to their ability to react with metallic cofactors (molybde-

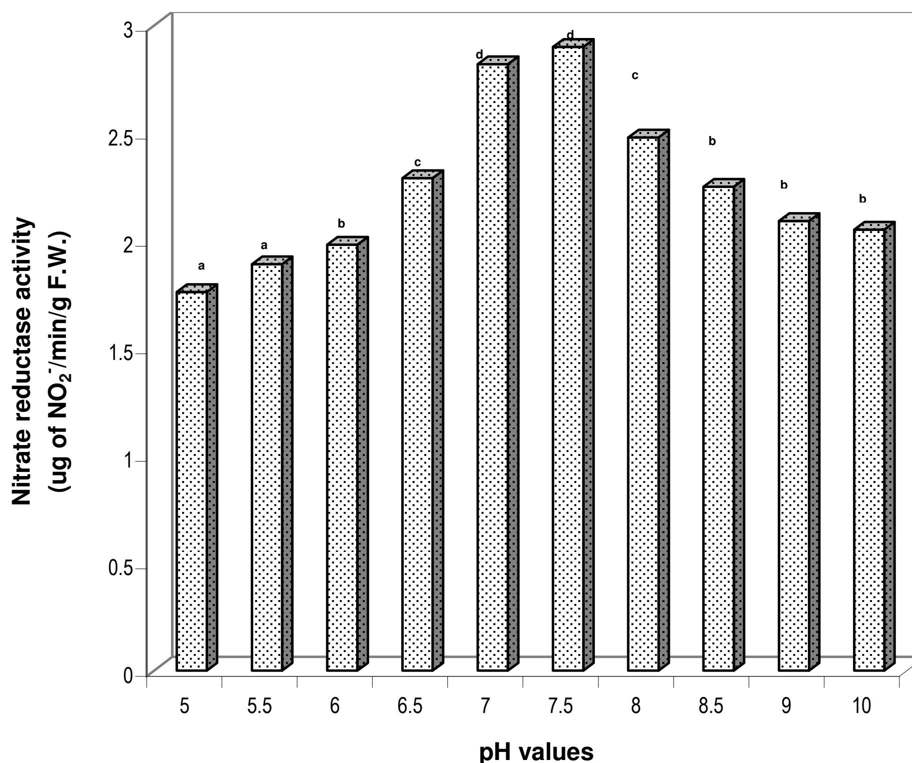

Figure 1. Influence of potassium phosphate buffer's pH values on cotton callus nitrate reductase activity. Values with the same letter are not significantly differents (Newman-keuls test at a = 5 %).

num and iron), which participate in the reduction of nitrate (Nason, 1963; Rosso et al., 1973; Buc, 1995). HEPES can also provide free radicals in the extraction medium for the inhibition of electron transfer involved in the reaction of nitrate reduction (Grady et al., 1988).

Sodium borate and sodium acetate buffer inhibit strongly cotton callus nitrate reductase activity. The presence of sodium in these buffers seems to induce a strong inhibition of the reduction reaction.

The best extraction buffer determined previously (potassium phosphate buffer) was used at various pH levels (pH 5 to 10). The highest nitrate reductase activities were obtained when enzyme was extracted with pH 7 and pH 7.5 buffers. These pH values gave on average the similar enzyme activities (Figure 1). The activity of the enzyme was however higher when it was extracted with pH 7.5 buffer. This pH value was chosen as the optimum buffer pH for the extraction of cotton nitrate reductase. Phosphate extraction buffer at pH 7.5 was also the most efficient pH buffer for the extraction of nitrate reductase of many plant species (Nakagawa et al*.,* 1985; Mori, 2000).

Stimulating effect of amino-acids in the extraction medium

The enzyme activity was significantly stimulated when glutamine was added to the extraction buffer (2.9 µg of NO_2^-/min/g F.W.) than when cysteine was added to the buffer medium (Figure 2). The most efficient glutamine concentration in the buffer was found to be 15 mM. Glutamine is known to control nitrate reductase activity in the leaves of certain plant species, through its action on nucleus transcription of the enzyme (Dzuibany et al., 1998). This regulatory effect of glutamine seems to continue even *in vitro*, during the enzyme activity measurement. Cysteine showed a stimulating effect when it was added to the reaction medium of corn nitrate reductase (Robin, 1979).

Reaction kinetic

Figure 3 shows a low nitrate reductase activity in cotton callus, when no exogenous substrate was added in the reaction medium. However, the most important production of nitrite by the enzyme was observed when 0.1 M of exogenous potassium nitrate was added to the reaction medium. Substrate concentration in the reaction medium affects the response of many enzymes. There is an optimum concentration of substrate, which gives the highest activity for each enzyme (Boyer, 1986). The same optimal concentration of substrate (0.1 M) was observed with nitrate reductase of *Anacardium occidentale* (Subbaiah and Balasimha, 1983), Table 1.

During enzyme activity measurement, the amount of nitrite produced under optimum conditions of extraction

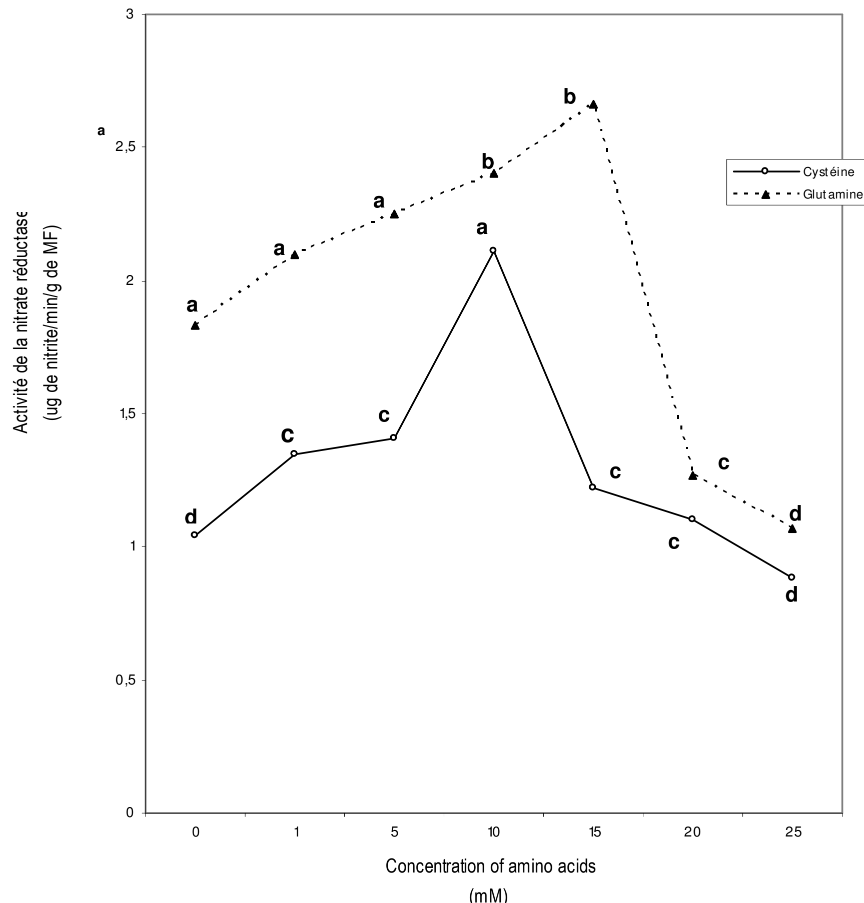

Figure 2. Influence of amino-acid concentrations in the extraction medium on cotton callus nitrate reductase activity. Values with the same letter are not significantly differents (Newman -Keuls test at a = 5%).

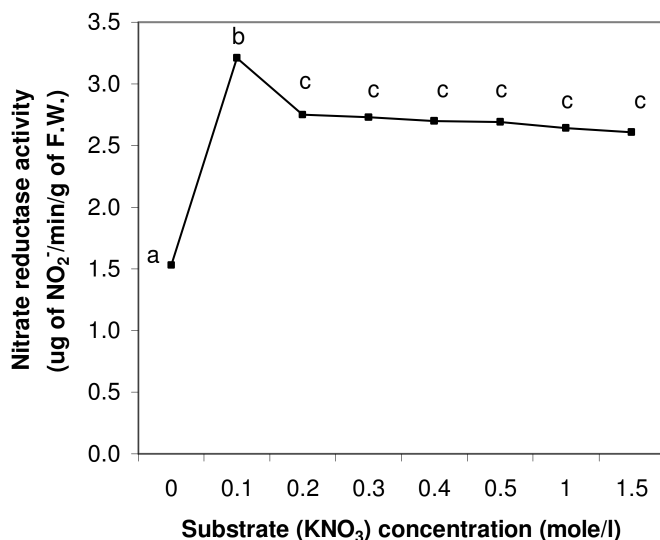

Figure 3. Effect of reaction medium nitrate concentration on cotton callus nitrate reductase activity. Values with the same letter are not significantly differents (Newman-keuls test at 5%).

and activities increased with the incubation period. The maximum production of nitrite occurred when the enzyme is incubated for 20 min. The production of nitrite remained constant for longer incubation periods (Figure 4). The substrate was entirely reduced after 20 min of incubation. On the other hand, Figure 5 shows that rateof nitrite production was faster for the shorter incubation period (1 to 5 min). The rate of nitrite production was, in this case, significantly higher (1.12 µg of NO_2^-/min/g F.W.) than that obtained with longer incubation periods. Reduction of nitrate is a spontaneous and reversible reaction, which is inhibited by the products of the reaction (NO_3^-) (Nason, 1963). Longer reaction time could thus reduce enzyme activity through the production of important quantities of nitrite.

CONCLUSION

Methods of nitrate reductase extraction used for other plant species do not give satisfactory results in term of enzyme activity, in cotton callus, because of the extreme

Table 1. Influence of the nature of the extraction buffer on cotton callus nitrate reductase activity

Buffer	potassium Phosphate	Tris-HCl	HEPES	Sodium borate	Sodium acetate
Nitrate reductase activity (µg of NO_2^-/min/g MF)	2,82 **a**	1,80 **b**	1,97 **b**	0,96 **c**	1,07 **c**

Values with the same letter are not significantly different (Newman-keuls test at α = 5 %).

Table 2. Influence of the incubation period length on the velocity of cotton nitrate reductase reaction.

Incubation period (min)	1	5	10	20	25	30	40
Activity (µg of NO2- g/min/g F.W)	1.12 **a**	0.36 **b**	0.27 **c**	0.19 **d**	0.15 **d**	0.13 **d**	0.025 **e**

Values with the same letter are not significantly different (Newman-Keuls test at a = 5 %).

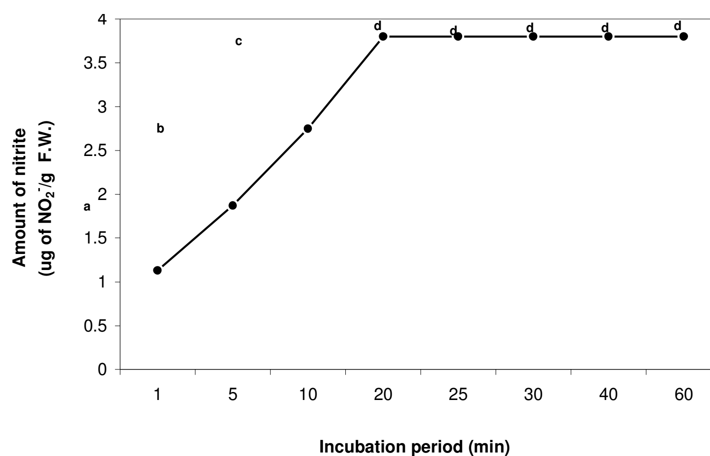

Figure 4. Nitrite production for different incubation periods of cotton callus nitrate reductase. Values with the same letter are not significantly different (Newman-keuls test at α = 5 %).

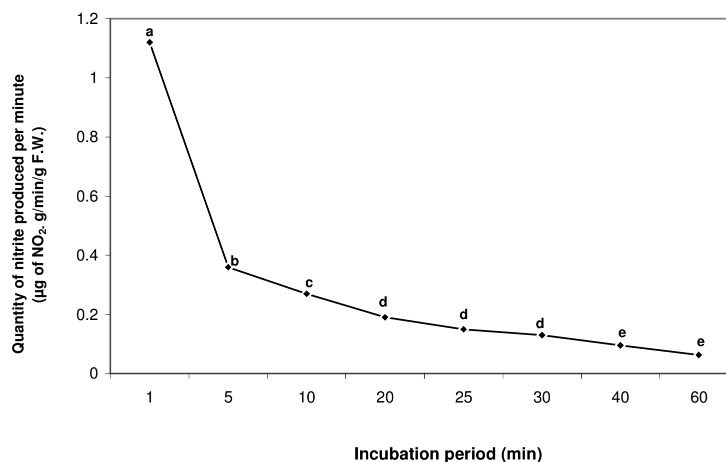

Figure 5. Influence of incubation time on cotton callus nitrate reductase activity. Values with the same letter are not significantly different (Newman-keuls test at α = 5 %).

variability in the enzyme response, according to plant species. According to our results, to obtain an optimum response of cotton nitrate reductase during activity measurements, the enzyme should be extracted with potassium phosphate buffer pH 7.5, containing 15 mM glutamine. Tris and HEPES, which are used for the extraction of nitrate reductase of other plant species, inhibited nitrite formation in cotton callus. Sodium contents of borate and acetate buffer also appeared to inhibit cotton nitrate reductase enzyme activity. Glutamine might control *in vivo*, the activity of nitrate reductase. This amino-acid also stimulated the activity of the enzyme *in vitro,* when it was added in the extraction solution*.* The reduction reaction showed an optimal concentration of substrate at 1 mM in the reaction medium and a higher activity when the incubation period of the enzyme was shorter (1 to 5 min).

ACKNOWLEDGEMENTS

The authors would like to thank the Development and Cooperation General Agency (DCGA) of Belgium for the financial aids provided, and the Laboratory of Tropical Crop Improvement of Catholic University of Leuven (Belgium) for its scientific support.

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