Characterization and Role of Glucose-6-phosphate Dehydrogenase of *Populus suaveolens* in Induction of Freezing Resistance

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ABSTRACT Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) was purified from the leaves of 8-week-old *Populus suaveolens* cuttings. The enzyme activity in the absence and presence of reduced dithiothreitol (DTT_{red}) was determined. The results show that the G6PDH activity is not inactivated by pre-incubation with DTT_{red}, indicating that the purified enzyme probably presented in cytosol of *P. suaveolens*. The catalytic characteristics and kinetic parameters of cytosolic G6PDH purified from *P. suaveolens* cuttings were also studied. The results show that G6PDH is characterized by K_m value of 360 μmol·L^{-1} for G6P and 16 μmol·L^{-1} for NADP, a pH range of 7.3–8.9, and the maximum activity around pH 8.2. The enzyme activity is inhibited by various metabolites such as NADPH, NADH, GTP, UTP, ATP, AMP, ADP, CoA, acetyl CoA, fructose-6-phosphate (F6P), erythrose-4-phosphate (E4P), ribose-5-phosphate (R5P) and 3-phosphoglycerate (3-PG) (all at 1 mmol·L^{-1} except for NADPH and NADH) to different extents. NADPH is the most effective inhibitor of enzyme activity, with an inhibition of 72.0%. The addition of metal ions such as $MgCl_2$, $CaCl_2$ and KCl (all 1.0 mmol·L^{-1}) to the standard reaction mixture has no remarkable influence on the cytosolic G6PDH activity. However, $CdCl_2$ (1.0 mmol·L^{-1}) causes high inhibitory effect on the enzyme activity. To explore the role of G6PDH on the enhancement of freezing resistance induced by freezing acclimation, the changes in the cytosolic G6PDH activity and freezing resistance (expressed as LT_{50}) of *P. suaveolens* cuttings during freezing acclimation at –20 °C were investigated. The results reveal that freezing acclimation decreases LT_{50} of cuttings, and increases the activity of cytosolic G6PDH compared with control ones, while 2 d of de-acclimation at 25 °C result in a decrease in cytosolic G6PDH activity, and caused an increase in LT_{50}. Furthermore, the change in cytosolic G6PDH activity is found to be closely correlated to the degree of freezing resistance of cuttings during freezing acclimation. It is suggested that cytosolic G6PDH may be involved in the induction of freezing resistance of cuttings.

KEY WORDS *Populus suaveolens*, freezing acclimation, freezing resistance, LT_{50}, G6PDH

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1 Introduction

Glucose-6-phosphate dehydrogenase (G6PDH) is the main regulated enzyme, which catalyzes the first irreversible reaction of the oxidative pentose phosphate pathway (OPPP), and its main physiological function is to provide NADPH for reductive biosyntheses (Copeland and Turner 1987, Graeve *et al*. 1994, von Schaewen *et al*. 1995, Dennis *et al*. 1997). In recent years, the studies of G6PDH have been focused on kinetic properties (Wendt *et al*. 2000, Semenikhina *et al*. 1999, 2001, Esposito *et al*. 2001, Hauschild and von Schaewen 2003), isoenzyme (Hong and Copeland 1991, Huppe and Turpin 1996, Esposito *et al*. 2001, Hauschild and von Schaewen 2003), subcellular distribution (Debnam and Emes 1999), the correlation between enzyme activity and nitrate or ammonia assimilation (Bowsher *et al*. 1992, Redinbaugh and Campbell 1998) and analysis of cDNA sequence (Graeve *et al*. 1994, von Schaewen *et al*. 1995, Schnarrenberger *et al*. 1995, Wenderoth *et al*. 1997, Wendt *et al*. 1999, 2000, Knight *et al*. 2001). But the above-mentioned studies only dealt with herbaceous plants.

It was first found that the G6PDH activity in poplar twigs exhibited a marked increase in fall and winter followed by a gradual decrease in spring (Sagisaka 1972). Sagisaka (1985) reported that G6PDH inactivation resulted in the injuries of poplar twigs during storage in a frozen state. In addition, an increase in G6PDH activity induced by low temperature was observed in alfalfa (Krasnuk *et al*. 1976), ryegrass (Bredemeijer and Esselink 1995), soybean (Van Heerden *et al*. 2003), banana (Lin *et al*. 2001), and *Populus tomentosa* (Lin and Zhang 2001). But up to now the actual role of G6PDH in the enhancement of freezing resistance induced by freezing acclimation in plants has not been clarified yet. Moreover, little is known about the correlation between the change in G6PDH activity and the degree of increase in freezing resis-

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tance induced by freezing acclimation, especially in woody plants.

Populus suaveolens, one of freezing resistant arbor plants, can survive under a temperature of approximately –43.5 °C in winter in the Great Xing'an Mountain, Northeast of China and consequentially is a better material to study the mechanism of freezing resistance of woody plants (Lin 2001, Lin *et al*. 2004). In the present study, the changes in G6PDH activity and LT_{50}, and the correlation mentioned above were investigated in detail using *P. suaveolens* cuttings to explore the role of G6PDH in the enhancement of freezing resistance induced by freezing acclimation. On the other hand, the catalytic characteristics and kinetic properties of G6PDH purified from *P. suaveolens* cuttings were studied in order to elucidate the physiological function and the regulation mechanism of G6PDH.

2 Materials and methods

2.1 Plant material

Populus suaveolens cuttings were obtained from Heilongjiang Province, China. The rooted cuttings grew in pots containing a 2:1 (v/v) mixture of soil and sand. After the cuttings grew for 5 weeks in a greenhouse, they were placed outdoors for 3 weeks, and used as experimental material.

2.2 Freezing acclimation and de-acclimation

Eight-week-old cuttings were divided into 3 groups. The first group of 100 cuttings placed at 25 °C was referred to as non-acclimated (NA or control) cuttings. The second group of 100 cuttings directly held at –20 °C for 6 d was referred to as freezing-acclimated (FA) cuttings, and some of the FA cuttings exposed to 25 °C for 2 d were referred to as de-acclimated (DA) cuttings. Cuttings of all treatments grew under an 8-h photoperiod and a light intensity of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

2.3 Evaluations of survival rates and LT_{50} of cuttings

All tested cuttings used for the evaluation of survival rates and LT_{50} were treated with different temperatures. Temperatures of –20, –23, –26, –29, –32 and –35 °C were used for NA and DA cuttings, –35, –38, –41, –44, –47, –50 and –53 °C used for FA cuttings. Cuttings were held at each temperature for 12 h. Then all tested cuttings were directly transferred from every treated temperature to 25 °C for 2 d. Survival cuttings were defined as an 80% of survival leaves and buds of cuttings after treatment at every temperature for 12 h, and then the amounts of survival cuttings were calculated. The percentage of survival cuttings is taken as survival rates. The data of survival rates of cuttings subjected to treatment with each temperature were used to fit exponential function by using the logarithm of survival rate as a liner function of the time of cold treatment, and then the temperature for 50% survival of cuttings (LT_{50}) was calculated. LT_{50} is taken as a measure of the freezing resistance.

2.4 Extraction, purification and analysis of G6PDH

G6PDH was extracted according to the procedure described by Graeve *et al* (1994) with some modification and its purity was tested by the method of Debnam and Emes (1999). 2 g of leaves from *P. suaveolens* cuttings acclimated at –20 °C for 6 d were ground under liquid nitrogen and suspended in 1.5 mL of an ice-cooled extraction buffer containing Tris-HCl 0.15 $mol \cdot L^{-1}$ (pH 8.0), NADP 0.1 $mmol \cdot L^{-1}$, PMSF 1 $mmol \cdot L^{-1}$, β-mercaptoethanol 3 $mmol \cdot L^{-1}$, 6-amino caproic acid 1$mmol \cdot L^{-1}$, benzamidine 2 $mmol \cdot L^{-1}$ and 2% (w/v) insoluble PVP. The homogenates were centrifuged at 4 °C with 16 000 *g* for 20 min and then solid ammonium sulfate was added to the supernatant to 40% of saturation. The solution was stirred for 30 min and precipitated proteins were removed by centrifugation for 30 min at 4 °C with 15 000 *g*. The resulting supernatant was collected, added with solid ammonium sulfate to 70% of saturation, and centrifuged as above. The insoluble material was dissolved in 2 mL of an ice-cooled buffer including Tris-HCl 0.03 $mol \cdot L^{-1}$ (pH 8.0), NADP 0.1 $mmol \cdot L^{-1}$, PMSF 1 $mmol \cdot L^{-1}$ and β-mercaptoethanol 5 $mmol \cdot L^{-1}$, 6-amino caproic acid 1 $mmol \cdot L^{-1}$ and benzamidine 2 $mmol \cdot L^{-1}$. Then the supernatant was used for the analysis of G6PDH. The activity of enzyme was measured in a butter containing Tris-HCl 0.15 $mol \cdot L^{-1}$ (pH 8.0), NADP 0.1 $mmol \cdot L^{-1}$ and G6P 3 $mmol \cdot L^{-1}$. The reaction was started by the addition of 30 μL enzyme preparation after 10 min of pre- incubation at 30 °C. One unit (U) of enzyme activity was defined as the amount of enzyme that increased a 0.01 of A_{340} per min under the assay condition.

The G6PDH in cytosol or in plastid is routinely distinguished by measuring G6PDH activity in the absence and presence of reduced dithiothreitol (DTT_{red}) under the standard reaction condition according to Johnson (1972).

The effect of various metabolites and metal irons (all at 1 $mmol \cdot L^{-1}$ except NADPH and NADH at 0.1 $mmol \cdot L^{-1}$) on the activity of G6PDH was measured at NADP 0.1 $mmol \cdot L^{-1}$ and G6P 3 $mmol \cdot L^{-1}$ in Tris-HCl

0.15 mol·L^{-1} buffer (pH 8.0) after 10 min of pre-incubation at 30 °C. In addition, to determine K_m and V_{max} values, the concentration of one substrate was held constant while the other one was allowed to vary.

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

All the determinations were performed at least in triplicates and the average values were presented.

3 Results

3.1 Effects of various metabolites and metal ions on the G6PDH activity

Effects of various metabolites and metal ions on the cytosolic G6PDH activity of *P. suaveolens* cuttings after 10 min of pre-incubation in the assay mixture at 30 °C are presented in Table 1. The G6PDH activity was inhibited about 72.0%, 44.2%, 31.5%, 25.3%, 53.2% and 38.5% by NADPH, GTP, UTP, ATP, CoA and acetyl CoA (all at 1.0 mmol·L^{-1} except NADPH), respectively. The following metabolites including ADP, AMP, NADH, fructose-6-phosphate (F6P), ribose-5-phosphate (R5P), erythrose-4-phosphate (E4P) and 3-phosphoglycerate (3-PG) (all at 1.0 mmol·L^{-1} except NADH) showed a smaller inhibitory effect on the G6PDH activity. The addition of metal ions such as $MgCl_2$, $CaCl_2$ and KCl (all 1.0 mmol·L^{-1}) to the standard reaction mixture had no remarkable influence on the G6PDH activity. However, $CdCl_2$ (1.0 mmol·L^{-1}) caused a high inhibitory effect on the enzyme activity (Table 1).

3.2 Effects of pH and DTT_{red} on G6PDH activity

After pre-incubation with DTT_{red} 70 mmol·L^{-1} for 100 min, G6PDH activity showed less than 5% decrease relative to the maximal activity of control (Fig. 1), indicating that pre-incubation with DTT_{red} had no effect on the G6PDH activity while the enzyme was in cytosol of *P. suaveolens*.

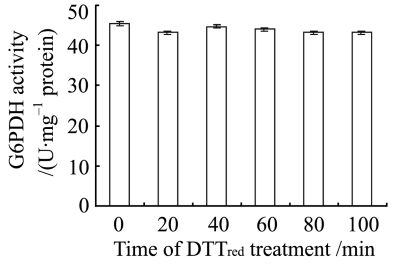

FIGURE 1 Effect of DTT_{red} on G6PDH activity

The measurement of cytosol G6PDH activity was performed using G6P 500 μmol·L^{-1} and NADP 40 μmol·L^{-1} as the substrates in the standard reaction mixture over a pH range of 7.0–9.2. G6PDH exhibited activity between pH 7.3 and 8.9, and showed maximum activity around pH 8.2 (Fig. 2).

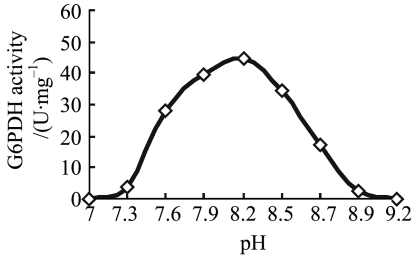

FIGURE 2 Effect of pH on G6PDH activity

TABLE 1 Effects of various metabolites and metal irons on the activity of G6PDH

Metabolite or metal ion	Concentration /(mmol·L^{-1})	Inhibition /%
None		0
NADPH	0.1	72.0 ± 2.2
CoA	1.0	53.2 ± 1.7
GTP	1.0	44.2 ± 1.2
Acetyl CoA	1.0	38.5 ± 1.3
UTP	1.0	31.5 ± 1.1
ATP	1.0	25.3 ± 1.6
AMP	1.0	10.4 ± 0.8
ADP	1.0	11.6 ± 1.0
NADH	0.1	12.8 ± 1.3
F6P	1.0	9.2 ± 0.6
3-PG	1.0	7.8 ± 0.5
R5P	1.0	7.0 ± 1.0
E4P	1.0	8.1 ± 0.9
$MgCl_2$	1.0	2.3 ± 0.5
$CaCl_2$	1.0	2.1 ± 0.7
KCl	1.0	1.5 ± 0.5
$CdCl_2$	1.0	89.1 ± 3.3

3.3 Kinetic parameters of G6PDH

The kinetic parameters K_m and V_{max} of cytosol G6PDH, obtained from Lineweaver-Burk plot, are shown in Figs. 3A and 3B. The K_m value for G6P was 360 μmol·L^{-1} at a saturation NADP concentration of 200 μmol·L^{-1} with variable G6P concentrations (1–50 μmol·L^{-1}) (Fig. 3A). Similarly, The K_m value of 16 μmol·L^{-1} for NADP was determined at a saturation G6P concentration of 50 μmol·L^{-1} with variable NADP concentrations (1–500 μmol·L^{-1}) (Fig. 3B). On the other hand, the V_{max} value of cytosol G6PDH was

estimated to be 68 U·mg^{-1} protein (Figs. 3A and 3B).

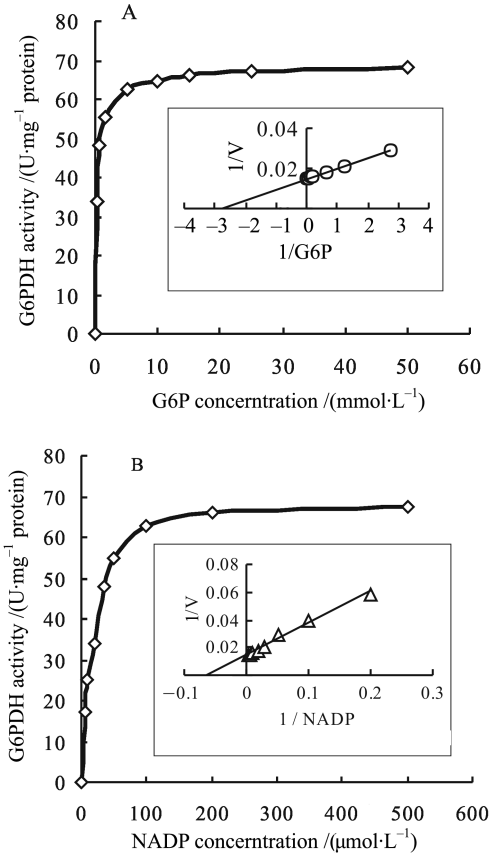

FIGURE 3 Effects of G6P and NADP on kinetic parameters of G6PDH

A: G6P as the variable substrate at 0.2 mmol·L^{-1} NADP; B: $NADP^+$ as the variable substrate at 0.04 mmol·L^{-1}

3.4 Change in freezing resistance

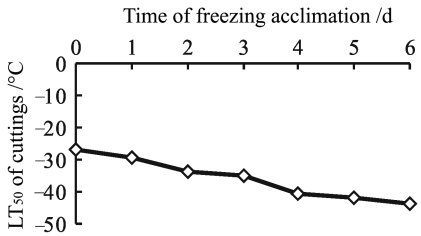

FIGURE 4 Change in LT_{50} of cuttings with time of freezing acclimation at –20 °C

After 6 d of freezing acclimation at –20 °C, the LT_{50} of *P. suaveolens* cuttings decreased from –27.1 °C for control cuttings to –43.5 °C for freezing acclimated ones. But after 2 d of de-acclimation at 25 °C, the LT_{50} of cuttings almost returned to the control level (Table 2). In order to demonstrate in detail the effect of freezing acclimation on freezing resistance of cuttings, the time course of freezing acclimation at –20 °C in a period of 1–6 d was studied (Fig. 4). One day after transferring cuttings to –20 °C, the LT_{50} of cuttings decreased gradually from –27.1 °C at the beginning to –43.5 °C on the 6th day.

TABLE 2 Changes in LT_{50} and G6PDH in *P. suaveolens* cuttings

Treatments	LT_{50} /°C	G6PDH /(U·mg^{-1} protein)
Control	–27.1	47.2
FA	–43.5	143.9
DA	–26.6	40.2

3.5 Change in G6PDH activity

The activity of cytosolic G6PDH of cuttings gradually increased with time of freezing acclimation in a period of 6 d (Fig. 5). After 6 d of freezing acclimation, the activity of cytosolic G6PDH increased about 2.0-fold compared with control cuttings, whereas 2 d of de-acclimation at 25 °C resulted in a decrease in cytosolic G6PDH activity (Table 2). In addition, the activity of cytosolic G6PDH increased with the decrease in LT_{50} of cuttings (Figs. 4, 5 and 6).

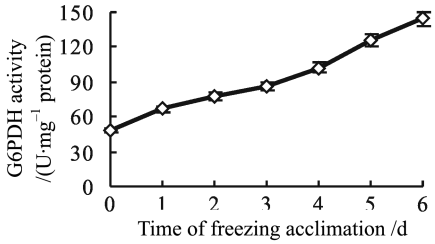

FIGURE 5 Effect of freezing acclimationat at –20 °C on G6PDH activity of cuttings

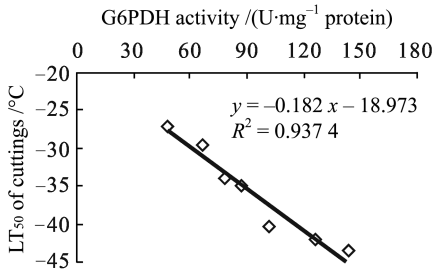

FIGURE 6 The relationship between G6PDH activity and LT_{50} in cuttings during freezing acclimation at –20 °C

4 Discussion

In plants, G6PDH activity is present in both cytosol and plastid (Dennis and Miernyk 1982). The G6PDH in cytosol or in plastid is routinely distinguished by measuring G6PDH activity in the absence and presence of reduced dithiothreitol (DTT_{red}) (Johnson 1972, Wendt *et al*. 2000). In this study, the G6PDH activity of *P. suaveolens* cuttings was not inactivated by pre-incubation with DTT_{red} (Fig. 1), as was the case for the cytosolic G6PDH of pea, spinach and potato (Fickenscher and Scheibe 1986, Graeve *et al*. 1994,

von Schaewen *et al*. 1995, Schnarrenberger *et al*. 1995, Wendt *et al*. 2000). Moreover, the G6PDH of *P. suaveolens* exhibited a pH range of 7.3–8.9 and the pH-optimum of the enzyme was observed at 8.2 (Fig. 2), similar to that of cytosolic G6PDH from potato (Hong and Copeland 1991, Graeve *et al*. 1994). The results showed that the purified G6PDH from *P. suaveolens* was probably present in cytosolic isoform.

The strong inhibition of cytosolic *P. suaveolens* G6PDH activity caused by NADPH was reported for G6PDH from soybean (Hong and Copeland 1991), pea (Fickenscher and Scheibe 1986), spinach (Lendzian 1980), black gram (Ashihara and Komamine 1976), barley (Esposito *et al*. 2001), and potato (Graeve *et al*. 1994). NADPH is the product of reaction catalyzed by G6PDH. The sensitivity of G6PDH to NADPH showed that the enzyme is strongly inhibited by its product in a competitive feedback inhibition manner. Wendt *et al*. (2000) reported that several influencing factors such as ATP, ADP, AMP, UDP, NADH, acetyl CoA, F6P, R5P and 3-PG had no effect on the G6PDH activity of potato. However, in our studies, the activity of G6PDH was inhibited by NADH, GTP, UTP, ATP, AMP, ADP, CoA, acetyl CoA, F6P, E4P, R5P or 3-PG to various extents (Table 1).

Graeve *et al*. (1994) reported that pre-incubation with Mg^{2+}, Ca^{2+} or K^{+} (each at 1.0 mmol·L^{-1}) had no remarkable influence on the potato G6PDH activity; similar findings were found in this study, indicating the unnecessity of Mg^{2+}, Ca^{2+} or K^{+} for the catalytic activity of G6PDH (Hauschild and von Schaewen 2003). However, at the same concentration, Cd^{2+} inhibited the G6PDH activity of *P. suaveolens* about 89.1% (Table 1), as was the case for pea G6PDH (Chugh and Sawhney 1999). The effect of Cd^{2+} on G6PDH activity is most likely to be though suppression of 6-phosphogluconate dehydrogenase systhesis and its adverse impact on G6PDH activity (Chugh and Sawhney 1999).

The affinity substrates of G6PDH are NADP and G6P. The K_m value of 260 μmol·L^{-1} for NADP and 6 μmol·L^{-1} for G6P were reported in cytosolic G6PDH from potato (Graeve *et al*. 1994). In our investigation, the cytosolic G6PDH activity of *P. suaveolens* was characterized by a K_m value of 360 μmol·L^{-1} for G6P and 16 μmol·L^{-1} for NADP (Figs. 3A and 3B); both K_m values were significantly higher than those of cytosolic G6PDH from potato (Graeve *et al*. 1994). The lower K_m value for NADP showed that cytosolic G6PDH had a high affinity towards NADP, being consistent with G6PDH of most all other plants (Mirfakhrai and Auleb 1989, Semenikhina *et al*. 2001). In addition, the observed V_{max} value of cytosolic G6PDH was about 68 U·mg^{-1} protein, which was higher than that of potato (65 U·mg^{-1} protein) (Graeve *et al*. 1994).

Although low temperature induced an increase of G6PDH activity as observed in some herbaceous plants, up to now reports concerning woody plants are very limited. Our previous studies found that a greater degree of increase in G6PDH activity and freezing resistance was observed in freezing-resistant cuttings of *P. suaveolens* than in freezing-sensitive cuttings of *P. tomentosa* during freezing acclimation (Lin and Zhang 2003). Moreover, the increase in G6PDH activity under low temperature was most probably of cytosolic origin (Lin and Zhang 2003, Van Heerden *et al*. 2003). In this study, we observed that freezing acclimation-induced increase in cytosolic G6PDH activity of *P. suaveolens* cuttings was followed by an obvious decrease after 2 d of de-acclimation at 25 °C (Table 2), indicating that the increase in cytosolic G6PDH activity during freezing acclimation might be in response to low temperature. Similar findings had been reported in ryegrass (Bredemeijer and Esselink 1995), soybean (Van Heerden *et al*. 2003), banana (Lin *et al*. 2001), and *P. tomentosa* (Lin and Zhang 2001). In addition, the LT_{50} in cuttings decreased with the increase in cytosolic G6PDH activity during freezing acclimation (Figs. 4 and 5), and there was a significant negative correlation between G6PDH activity and LT_{50} (r = –0.968**) (Fig. 6). The results showed that the increased cytosolic G6PDH of *P. suaveolens* cuttings might be dependent on freezing acclimation, which might be related to the enhancement of freezing resistance of cuttings.

It has been reported that the response of plants to low temperature is the effect on the pentose phosphate pathway (PPP) and its key regulatory enzyme (G6PDH), which are required for the induction of freezing resistance (Sadakane and Hatano 1982, Bredemeijer and Esselink 1995, Van Heerden *et al*. 2003). In general, the main physiological function of G6PDH is to provide NADPH for anabolic metabolisms and detoxifation reactions. In addition, PPP also generates some important intermediate metabolites such as pentoses, erythose-4P and ribose 5-phosphate for the synthesis of nucleotides and aromatic amino acids (Copeland and Turner 1987, Graeve *et al*. 1994, Dennis *et al*. 1997, Van Heerden *et al*. 2003, Lin and Zhang 2003). Thus, it was suggested that the increased activity of cytosolic G6PDH of *P. suaveolens* cuttings caused by

freezing acclimation enhance the capacity of OPPP to generate NADPH, which further result in the adaptive changes associated with the development and enhancement of freezing resistance of cuttings.

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Rural Fuelwood Markets in Niger

— An assessment of Danish Support to the Niger Household Energy Strategy 1989-2003

Dolf Noppen, Paul Kerkhof and Ced Hesse

During a period of almost fifteen years, Danish development cooperation has been involved in the fuelwood supply sector in Niger, first through the World Bank (Project Energie II, 1989-1998), and subsequently bilaterally, through the Household Energy Project (Project Energie Domestique, 2000-2003). Taken together, these initiatives have contributed to the development of an approach known, in Niger, as the Household Energy Strategy — a strategy which, although not a legal document, is nonetheless supported by a number of legal instruments which formalize rural fuelwood markets and which have as their objective the establishment of decentralized management of fuelwood and the financial procedures necessary for this to function.

This study is an assessment of the results of this long period of involvement and is aimed at others working with natural resources management both in the Sahel as well as elsewhere. The Niger experience has already prompted similar approaches in other countries within the region (Mali, Chad, Burkina Faso) as well as further afield (Madagascar, Mozambique). The study also supports the consolidation and further development of the Household Energy Strategy in Niger.

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