Effects of sodium nitroprusside on activity of acid and alkaline invertases and alkaline phosphatase in lemongrass (*Cymbopogon flexuosus Steud*) Wats

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Abstract

We investigated effects of sodium nitroprusside (SNP) on sucrose metabolizing enzymes, acid, and alkaline invertase and alkaline phosphatase in lemongrass (*Cymbopogon flexuosus* Steud) Wats varieties i.e. Krishna, Cauveri, Nima and Cheerharit. Fifteen day old lemongrass tillers were treated with SNP (1 and 2 mM) under sunlight for four hours. Our results clearly indicated that SNP (2 mM) substantially decreased the amount of proteins in all varieties studied, with maximum values of 40% and 33% in Nima and Krishna, respectively. SNP (1 mM) significantly increased the amount of proteins 43% and 31% in Krishna and Cauveri, respectively. SNP (2 mM) rapidly and severely reduced the activity of acid and alkaline invertases in all varieties, except Krishna and Cauveri. However, the effect of SNP was more pronounced on acid invertase causing at 2 mM an inhibition of 37%, 35% and 28% in Cheerharit, Nima and Cauveri, respectively, whereas it showed relatively less inhibition in alkaline invertase activity 27%, 24% and 21%, respectively, in Nima, Krishna and Cheerharit. Alkaline phosphatase activity was only considerably decreased following SNP (2 mM) treatment in all lemongrass varieties studied with the exception of Nima, where a sharp decrease of 50% was observed. SNP (1 mM) also demonstrated similar effects on acid and alkaline invertases and alkaline phosphatase. These results clearly suggest that SNP affects acid and alkaline phosphatase activity and, therefore, has a role in sucrose metabolism in lemongrass. Alterations in alkaline phosphatase activity upon SNP treatment have several consequences.

Introduction

Sodium nitroprusside (SNP) has long been used as a nitric oxide (NO) donor in plants. It has been regarded as an important intermediate and intracellular signaling molecule in plants that mediates various physiological and developmental processes, including expression of defence related genes and programmed cell death, stomata closure, seed generation and root development.[1-6] A number of earlier and current reports have described inhibitory effects of NO on net photosynthesis in a variety of plants i.e. oat (*Avena sativa*) and alfalfa (*Medicago sativa*).[7] Very recently, Lum *et al.*[8] have reported effects of sodium nitroprusside on the level of photosynthetic enzymes and glucose metabolism in mung bean (*Phaseolus aureus*) leaves. Beside effects of SNP on primary metabolism/photosynthesis, it is also reported to affect the level of secondary metabolites in plants. Our very recent study has revealed that SNP affects the level of anthocyanin and flavonol glycosides in pea (*Pisum sativum* L. cv. Arkel) leaves.[9]

Cymbopogon flexuosus (Steud) Wats, commonly known as lemongrass (Poaceae family), is a commercial source of an essential oil that has immense value in the flavorings, fragrance, cosmetics and perfumery industries.[10,11] Several previous studies on *C. flexuosus* have provided insight into the chemical composition of the essential oil, its biosynthesis and regulation, and accumulation etc.[12,13] From earlier studies on *C. flexuosus* and *C. martinii* (palmarosa), it has become very clear that there is a direct correlation between essential oil biosynthesis and sucrose mobilization[14,15] as well as oxidative pathways or primary metabolism.[13,16] Therefore, it is obvious that any interference in sucrose metabolism will result in a decrease in essential oil biosynthesis and accumulation. The aim of the present study was to investigate the effects of SNP on sucrose metabolism by assessment of the activities of acid and alkaline invertases that hydrolyze sucrose into glucose and fructose. Also, we are keen to know whether nitric oxide has a signaling role between sucrose metabolism and essential oil (secondary product) biosynthesis in lemongrass. To the best of our knowledge, this is the first study of SNP in lemongrasses in relation to sucrose metabolism. However, the present study is limited to the effects of SNP on sucrose metabolizing enzymes and alkaline phosphates; studies aimed at revealing the direct role of NO in essential biosynthesis via sucrose metabolism are ongoing.

Materials and Methods

Plants

Cymbopogon flexuosus (Steud) Wats varieties Krishna, Cauveri, Nima and Cheerharit tillers were grown in the Herbal Garden, Vellore Institute of Technology (VIT) University, Vellore, Tamil Nadu, India by following standard agronomic practices.

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SNP treatment of the lemongrass tillers

Fifteen day old lemongrass tillers were harvested and immediately dipped in round bottom tubes containing 5 mL SNP solution of variable dilutions (0, 1, 2 and 5 mM SNP). The tubes were then kept in mild sunlight for four hours for complete uptake of the SNP solution. This was achieved after two hours. The tubes were then kept filled with half-strength Hoagland solution.[17] After four hours of incubation, tillers were removed and leaves were separated, cut into pieces, weighed and used for the estimation of chlorophyll, and extraction of enzymes. SNP solutions were freshly prepared immediately before each experiment.

Chlorophyll estimation

Chlorophyll content of the non-treated (control) and SNP treated leaves were estimated by the method of Arnon.[18]

Extraction and assay of acid and alkaline invertase

Acid and alkaline invertases (EC 3.2.1.26 and 3.2.1.153) were extracted and assayed according to Singh and Luthra.[14] SNP treated leaves (3 g) were cut into small pieces and homogenized using pestle and mortar in 50

mM Tris-maleate buffer, pH-7.5 (1g leaf tissue: 3 mL buffer), 20 mM $MgCl_2$, 1 mM β-mercaptoethanol. The homogenate thus obtained was squeezed through four layers of muslin cloth and centrifuged at 15,000 rpm at 4ºC for 45 min. The supernatant was collected and used for enzyme assay. Protein content in the extract was estimated by the method of Bradford.[19]

For the assay of acid invertase, leaf protein extract (1 mg) along with the substrate 10 mM sucrose were mixed in 0.2 M sodium acetate buffer, pH 4.8 and incubated at 30ºC for 30 min. The reaction was stopped by addition of alkaline cooper reagent. Hydrolysis products of sucrose, invert sugars were estimated by the method of Nelson.[20] A similar assay system with, however, 0.2 M phosphate-citrate buffer pH 7.5 was used for alkaline invertase.

Estimation of invert sugars

Liberated invert sugars in the assays system was estimated by the method of Nelson.[20] The enzymic reaction was stopped by addition of 1 mL alkaline copper reagent to the test tubes. Contents of the tubes were thoroughly mixed and kept in a boiling water bath for 20 min. After incubation, tubes were removed from the boiling water bath and placed in cold water. After cooling the tubes, 1 mL of arsenomolybdate reagent was added and thoroughly mixed. The tubes were repeatedly shaken for 5 min until Cu_2O was completely dissolved. After this, 7 mL of distilled water were added and contents were vortexed and subjected to spectrometric analysis at 620 nm.

Assay of alkaline phosphatase

Assay system for alkaline phosphatase (EC 3.1.3.1) consisted of 100 mM glycine buffer, pH 10.4, 10 mM $MgCl_2$, 15 mM p-nitrophenyl phosphate (p-NPP) as substrate, and enzyme extract (1 mg). The assay mixture was incubated at 37ºC for 10 min. Reaction was stopped by the addition of 10 mL, 20 mM NaOH. Paranitrophenol (pNP) produced from pNPP was measured at 410 nm.

Results

Effect of SNP on chlorophyll content

Prior to the estimation of chlorophyll content and enzyme activities, cell viability in terms of approximately 50% cell death was checked following SNP treatment as described by Lum *et al.*[8] After SNP treatment, leaf sections were prepared, treated with 0.1% trypan blue for 10 min then washed thoroughly with distilled water to remove excess stain and observed for cell death under a light microscope. Cells stained blue represented dead cells. Our results on cell viability demonstrated that SNP (1 and 2 mM) did not cause cell death during the four hour incubation period; however, SNP (5 mM) caused 50% cell death during the same time period. Therefore, only 1 and 2 mM SNP concentrations were used to asses their effects on the chosen parameters.

Table 1. Effects of sodiumnitroprusside (SNP) on chlorophyll contents in *Cymbopogon flexuosus* varieties Krishna, Cauveri, Nima and Cheerharit. A 15 d lemongrass tiller was dipped in a 30 mL capacity culture tube containing 5 mL SNP solution of variable concentration (1-2 mM) and exposed under sunlight for 4 h. Three independent reading were recorded and values were expressed as the mean (± SD).

Treatment	Chlorophyll (mg . g FW^{-1})			
	Krishna	Cauveri	Nima	Cheerharit
No SNP	1.35±0.05	1.17±0.06	1.37±0.05	1.16±0.10
1mM	1.29±0.10	1.14±0.15	1.30±0.10	1.17±0.10
2 mM	1.18±0.10	1.00±0.05	1.20±0.10	0.94±0.06

Table 2. Effect of sodium nitroprusside (SNP) on leaf protein content in *Cymbopogon flexuosus* varieties, Krishna, Cauveri, Nima and Cheerharit. A 15 d lemongrass tiller was dipped in a 30 mL capacity culture tube containing 5 mL SNP solution of variable concentration (1-2 mM) and exposed under sunlight for 4 h. Three independent readings were recorded and values were expressed as the mean (± SD).

Treatment	Protein (mg . g FW^{-1})			
	Krishna	Cauveri	Nima	Cheerharit
No SNP	0.39±0.10	0.58±0.05	1.5±0.05	1.7±0.10
1 mM	0.56±0.13	0.76±0.15	1.9±0.49	1.9±0.37
2 mM	0.26±0.13	0.48±0.15	0.9±0.49	1.5±0.37

Chlorophyll contents of the non-treated leaves in the four varieties studied ranged from 1.16-1.37 mg/g fresh weight. However, results presented in Table 1 clearly show that chlorophyll content decreased considerably up to 20-25% from normal levels following SNP treatment.

Effect of SNP on total protein content

Protein content of the non-treated and SNP treated leaves are shown in Table 2. SNP (2 mM) causes a rapid and significant decrease in protein content of leaves in all the varieties of lemongrass studied. SNP (2 mM) treatment in the Nima and Krishna leaves caused a drastic reduction in protein content; 40 and 33%, respectively, while this was comparatively less in Cauveri and Cheerharit at 17% and 12%, respectively. However, surprisingly, a sharp increase in protein content of all the tested leaves was observed following 1 mM SNP treatment. After treatment with 1 mM SNP, the amount of protein in Krishna leaves was increased by 44%, Cauveri 31%, Nima 27% but only by 12% in Cheerharit.

Effects of SNP on acid and alkaline invertases and alkaline phosphatase

Our results demonstrated that SNP (1 and 2 mM) rapidly and substantially decreases activities of both acid and alkaline invertases in all the varieties, except Krishna and Cauveri where effects were haphazard. It was noted that the degree of inhibition varies with the concentration of SNP used. Effects of SNP were more pronounced on acid invertase activity; SNP (2 mM) markedly inhibited the activity of acid invertase by 37% in Cheerharit, 35% Nima and 28% Cauveri compared to non-treated leaves (Table 3A). In contrast, sodium nitroprusside (2 mM) had less effect on alkaline invertase activity; SNP (2 mM) resulted in a significant loss in the activity of alkaline invertase with 21% in Cheerharit, 28% Nima and 24% Krishna. SNP at lower concentration (1 mM) was also found to be equally effective causing noted inhibition in acid invertase activity; 20% in Krishna, 19% Cheerharit, 18% Nima and 16% Cauveri. Inhibition due to 1 mM SNP in alkaline invertase activity was 22% in Cauveri, 18% Nima, 16% Krishna and 12% Cheerharit (Table 3B).

SNP has relatively less effect on the activity of alkaline phosphatase compared to acid and alkaline invertase activities in the lemongrass varieties studied here (Table 4). SNP (2 mM) has significantly decreased the activity of alkaline phosphatase in Nima and Krishna causing inhibition of 29% and 50%, respectively, whereas in Cauveri and Cheerharit the extent of inhibition was only around 18% (Figure 1).

Discussion

The present study focuses on the effects of nitric oxide (NO) released from SNP on chlorophyll, protein content, sucrose metabolizing

enzymes, acid and alkaline invertase and alkaline phosphatase in four different varieties of *C. flexuosus*. For this study, we chose 15 day old lemongrass tillers because the tillers are growing and developing rapidly, and tillers are metabolically most active since rates of photosynthesis, sucrose breakdown and essential oil biogenesis are at the highest level. Sodium nitroprusside liberates NO, an important signaling molecule with multifunctional roles in plant life. One of the important roles of SNP is linked with chlorophyll biosynthesis and chloroplast development in plants grown under iron-deficient conditions. Basically, SNP influences iron metabolism, transport and availability in plants. In the present study, SNP has considerably reduced chlorophyll content in the lemongrass varieties studied; these results contrast with those reported previously in iron-deficient maize and potato leaves where SNP tended to increase the chlorophyll content. The comparatively very high concentration of SNP used in the present study could be a reason for depletion in chlorophyll content.

Sucrose, a disaccharide composed of glucose and fructose, is essential for plant growth and development. Sucrose and starch metabolism in *Cymbopogon* species is very well understood. In *C. flexuosus* and *C. martinii*, breakdown and mobilization of starch and sucrose has been correlated with essential oil biosynthesis.[14,15] The activity of acid invertase, a major sucrose metabolizing enzyme has been recorded to be at a maximum while alkaline invertase was relatively less active during early leaf/inflorescence development in *Cymbopogon* species. Since sucrose metabolism is well understood, we decided to check the effects of SNP on two sucrose metabolizing enzyme acid and alkaline invertases. Our study revealed that SNP liberated NO inhibited acid invertase more than alkaline invertase (Figure 1) in the lemongrass varieties studied. Inhibition of acid and alkaline invertase consequently stops sucrose breakdown which may have adverse effects on growth and development and essential oil biosynthesis. However, further studies will be needed to clarify this. From the present study, the mechanism of inactivation of the two invertases is not clear; perhaps detrimental effects of SNP on enzyme structure can be the cause of a decrease in invertase activity. As yet, there have been no studies with SNP on *Cymbopogon* species examining sucrose breakdown so there is no basic information available on the after effects of SNP on sucrose metabolism. However, the present study signals the role of NO in sucrose metabolism in lemongrass.

Allkaline phosphatases are ubiquitous in plants and mediate many biochemical reactions in cells. Sodium nitroprusside has demonstrated inhibitory effects on alkaline phosphatase in the lemongrass varieties stud-

Table 3A. Effect of sodium nitroprusside (SNP) on acid invertase in *Cymbopogon flexuosus* varieties, Krishna, Cauveri, Nima and Cheerharit. A 15 d lemongrass tiller was dipped in a 30 mL capacity culture tube containing 5 mL SNP solution of variable concentration (1-2 mM) and exposed under sunlight for 4 h. Three independent readings were recorded and values were expressed as the mean (± SD).

Treatment	Enzyme activity (µmol glc.hr^{-1}g FW^{-1})			
	Krishna	Cauveri	Nima	Cheerharit
No SNP	15±1.00	25±1.50	34±2.00	16±1.00
1 mM	12±1.91	21±2.50	28±4.58	13±2.29
2 mM	14±1.91	18±2.50	22±4.58	10±2.29

Table 3B. Effects of sodium nitroprusside (SNP) on alkaline invertase in *Cymbopogon flexuosus* varieties Krishna, Cauveri, Nima and Cheerharit. A 15 d lemongrass tiller was dipped in a 30 mL capacity culture tube containing 5 mL SNP solution of variable concentration (1-2 mM) and exposed under sunlight for 4 h. Three independent readings were recorded and values were expressed as the mean (±SD).

Treatment	Enzyme activity (µmol glc.hr^{-1}g FW^{-1})			
	Krishna	Cauveri	Nima	Cheerharit
No SNP	25±2.00	18±1.00	40±1.00	43±1.00
1 mM	21±1.68	14±1.48	33±4.30	38±3.34
2 mM	19±1.68	17±1.48	29±4.30	34±3.34

Table 4. Effect of sodium nitroprusside(SNP) on alkaline phosphatase activity in *Cymbopogon flexuosus* varieties Krishna, Cauveri, Nima and Cheerharit. A 15 d lemongrass tiller was dipped in a 30 mL capacity culture tube containing 5 mL SNP solution of variable concentration (1-2 mM) and exposed under sunlight for 4 h. Three independent readings were recorded and values were expressed as the mean (±SD).

Treatment	Alkaline phosphatase (units mL^{-1})			
	Krishna	Cauveri	Nima	Cheerharit
No SNP	0.21±0.02	0.55±0.01	0.50±0.01	0.30±0.01
1 mM	0.27±0.06	0.49±0.11	0.35±0.12	0.27±0.03
2 mM	0.15±0.06	0.45±0.11	0.25±0.12	0.25±0.03

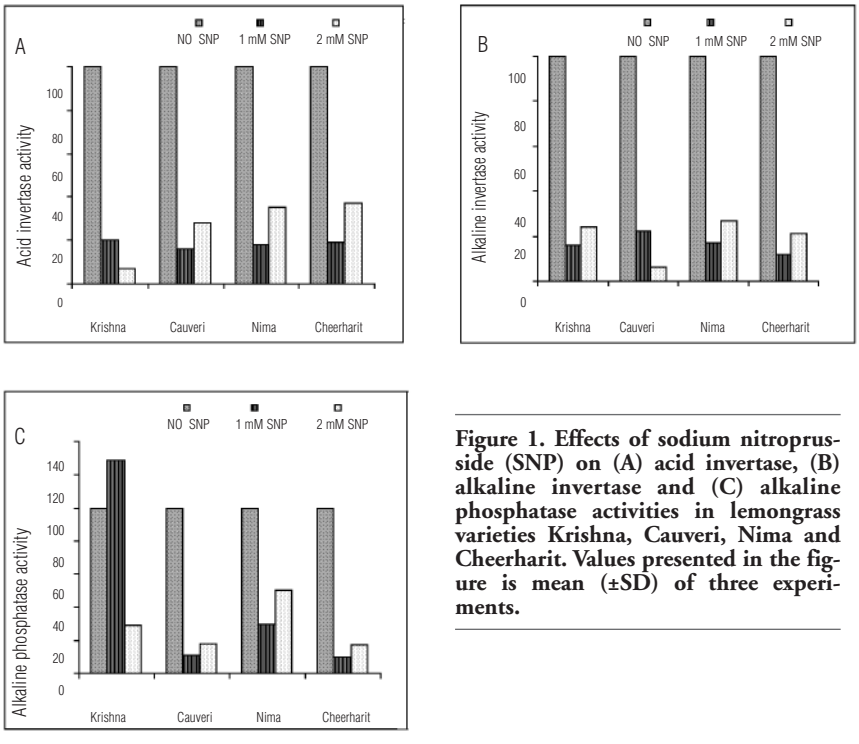

Figure 1. Effects of sodium nitroprusside (SNP) on (A) acid invertase, (B) alkaline invertase and (C) alkaline phosphatase activities in lemongrass varieties Krishna, Cauveri, Nima and Cheerharit. Values presented in the figure is mean (±SD) of three experiments.

ied (Figure 1). Inhibition of alkaline phosphate activity may lead to several biochemical and physiological changes in cells in view of its ubiquitous nature. In conclusion, the study suggested the role of NO on sucrose breakdown in lemongrass. A further study on the possible role of nitric oxide in sucrose metabolism and essential oil biosynthesis is ongoing.

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