Effect Of Panchagavya On Nitrate Assimilation By Experimental Plants

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ABSTRACT - Panchagavya, a vedic formulation for increased productivity and disease resistance in plants and a modified formulation amended with seaweed extract (*Sargassum wightii*) have been investigated for their effect on the enzymes of nitrate reduction and assimilation namely, NR, NiR, GS, GOGAT and GDH in the leaves of the seedlings of the pulses, *Vigna radiata, Vigna mungo, Arachis hypogaea, Cyamopsis tetragonoloba, Lablab purpureus, Cicer arietinum* and the cereal *Oryza sativa* var. *ponni*. The seaweed based panchagavya formulation increased the levels of all the enzymes in the experimental plants when used as manure at low concentrations i.e 1: 50 and 1: 100 (panchagavya: soil). Traditional panchagavya at 1: 100 dilutions was able to exhibit an increase in the levels of NR and NiR only. The enzymes GS, GOGAT and GDH did not show any response to the use of traditional panchagavya formulation when used as manure. [The Journal of American Science. 2010;6(2):80-86]. (ISSN 1545-1003).

***Keywords*:** Panchagavya, seaweed based, NR, NiR, GS, NAD(P)H-GOGAT, NAD(P)H-GDH, Pulses, Rice.

INTRODUCTION

There is growing concern over the current agricultural practices in terms of sustainability over long periods since it might cause a gradual decline in factor productivity with adverse impact on soil health and quality (Subba rao, 1999; Stockdale, 2000). It has been widely accepted that organic farming alone could serve as a holistic approach towards achieving sustainable agriculture as it is nature based, environment friendly and ensures the conservation of resources for the future. Organic farming is quite distinct in the sense that it relies on closed nutrient cycles with less dependence on off-farm inputs. Vedic literatures (*Vrikshayurveda*) have clearly outlined a systematized agricultural practice that insisted on the use of 'panchagavya' – a mixture of the five products of cow in a specific ratio to enhance the biological efficiency of crop plants and the quality of fruits and vegetables (Natarajan, 2002). Few farmers in the Southern parts of India are using a modified preparation of panchagavya in organic farming (Gomathynayagam, 2001). In recent years, the crude extracts of seaweeds or the marine macroalgae have been shown to possess biostimulant, biofertilizer and antimicrobial properties (Borowitzka and Borowitzka, 1988; Robles-Centeno and Ballantine, 1999; Selvaraju, 2002) and, many commercial preparations are available in the market under different brand names. Seaweed extracts contain minerals, vitamins, free aminoacids and polyunsaturated fatty acids in addition to growth hormones (Yamamato *et al*., 1975; Tay *et al*., 1985; Dave *et al*., 1993; Thevanathan *et al*., 1993; Tasneem Fatima *et al*., 1994; Selvi *et al*., 1999). The combined effect of panchagavya and seaweed extract on the growth and productivity of crop plants is however, not known. In this paper, we present the results of a study on the nitrate assimilation in the seedlings of some pulses and rice grown in a soil preparation amended with seaweed based panchagavya as manure.

MATERIALS AND METHODS

Experimental plants

The pulses *Vigna radiata, Vigna mungo, Arachis hypogaea, Cyamopsis tetragonoloba, Lablab purpureus, Cicer arietinum* and the cereal *Oryza sativa* var. *ponni* were used as the experimental plants.

Preparation of soil and panchagavya for the growth of experimental plants

Traditional and seaweed based panchagavya were prepared as described (Sangeetha, 2009). Both preparations of panchagavya were brought to a solid state and the

dried panchagavya was mixed with garden soil and used for planting the seeds of the experimental plants. Traditional panchagavya was used in the ratio of 1: 100 (traditional panchagavya: soil) and the seaweed based panchagavya at a ratio of 1: 50 (v/v) and 1: 100 (v/v).

Seeds were surface sterilized with 1.0% mercuric chloride, washed several times in running water, soaked overnight in sterile water and allowed to germinate in dark. Germinating seeds were implanted in soil preparations kept in pots of the size 5.2" tall and 3.5" radius. Seedlings raised in sterilized garden soil were used as control. Ten replicates were used for all experimental plants. The seedlings of pulses were inoculated with *Rhizobium* R_4 (approximately $1x10^9$ cells/mL of sterile nutrient solution) twice, i.e. on the 5th day and then on the 11th day in both control and test pots (except for *Oryza sativa* var. *ponni*). *Rhizobium* (R4) was maintained on YMA medium. All the developing seedlings except *Oryza sativa* were watered on alternate days with Wilson's N-free nutrient solution (Wilson and Reisnauer, 1963). For *Oryza sativa*, normal water was used. Twenty one days old seedlings were carefully removed and cell free enzymes were used for the assay of the enzymes.

Extraction of cell-free enzymes

Freshly harvested leaves were rinsed in ice-cold, sterile water and homogenized with 5.0 mL of ice-cold Marsden's buffer, pH 7.4 containing 50.0 mM MOPS [3-(N-morpholino) propanesulfonic acid)], 2.0 mM EDTA, 50.0 mM ascorbic acid, 0.5 mM dithiothreitol, $CaCl_2$ (0.2 g L^{-1}), TWEEN 80 (1.0 mL L^{-1}) and insoluble polyvinyl pyrrolidone (PVP) (100 g L^{-1}) (pretreated according to Loomis, 1974). The homogenate was strained through three layers of cheese cloth and centrifuged at 7000 x g for 15 minutes. The supernatant was collected separately and centrifuged at 20,000 x g for 30 minutes. The supernatant (crude extract) thus obtained was treated with Sephadex G-25 and dialyzed overnight. The dialysate was centrifuged at 20,000 x g for 30 minutes. The resulting clear supernatant was used as the enzyme extract (Thevanathan, 1980). The entire operation was carried out at 4°C. The extraction procedure is same for all the enzymes, unless otherwise mentioned.

Assay of enzymes

Nitrate Reductase (NR; E.C. 1.6.6.1): The *in vitro* NR levels in the enzyme preparation were assayed following the procedures of Thomas and Harrison (1988). The reaction mixture for *in vitro* assay of NR in a total volume of 2.0 mL contained 0.2 mL of 0.1 M KNO_3, 0.3 mL of 0.8 M $MgSO_4$, 0.5 mL of 720 µM NADH and 1.0 mL of the enzyme extract. The reaction mixture was incubated at 30°C for 30 minutes and the reaction was stopped by the addition of 5.0 mL of ice cold 95% ethanol and 0.2 mL of 1.0 M Zinc acetate. The resultant suspension was centrifuged at 2000 x g for 5 minutes, and the nitrite content of the supernatant was determined by the addition of 1.0 mL sulphanilamide (0.2% W/V) followed within two minutes by 1.0 mL of N (1-napthyl) ethylene diamine dihydrochloride (NEDD) (0.05% W/V) (Snell & Snell, 1949). The absorbance of the solution was measured at 543 nm. Controls lacked NADH. Enzyme activity was expressed as n mole nitrite formed per gram protein per hour.

Nitrite Reductase (NiR; E.C. 1.6.6.4): *In vitro* NiR levels were assayed by a modified method of Joy and Hageman (1966). The reaction mixture in a total volume of 2.0 mL contained 100 µ moles potassium phosphate buffer (pH 7.5), 1.5 µ moles potassium nitrite, 1.0 µ moles methyl viologen dye and 0.2 mL of the enzyme extract. The reaction mixture was kept over ice, over layered with a thin layer of liquid paraffin to prevent rapid oxidation of chemically reduced methyl viologen and then incubated in a water bath at 30°C. The reaction was then initiated by pipetting freshly prepared sodium dithionite below the paraffin layer. The contents were gently stirred to ensure uniform distribution of the reduced dye and incubated at 30°C for 30 minutes. Reaction was terminated by shaking the contents vigorously till the disappearance of blue color. Reagent blank and boiled enzyme control were run simultaneously. After the termination of reaction, 2.0 mL of distilled water was added to the reaction mixture. Residual nitrite was determined by diazotization (Snell & Snell, 1949) as described earlier for nitrate reductase except that 0.1 mL aliquots were removed and made up to 3.0 mL with distilled water. The absorbance was measured at 540 nm against substrate blank. Nitrite reductase activity was expressed as n mole NO_2^- consumed per minute per gram protein.

Glutamine synthetase (GS; E.C. 6.3.1.2): Glutamine synthetase was assayed by the γ-glutamyl hydroxamate assay of Shapiro and Stadtman (1970), with the exception that the pH of the two-fold assay mixture was changed to 7.4 (Thevanathan, 1980). The the two-fold assay mixture in a final volume of 25.0 mL contained 2.0 mL of 1.0 M imidazole (pH 7.4), 15.0 mL of 0.1 M

glutamine (pH 7.4), 1.5 ml of 0.01 M $MnCl_2.H_2O$, 0.06 mL of 1.0 M $MgCl_2$, 2.0 mL of 0.01 M ADP – trisodium salt, 1.0 mL of 1.0 M $Na_2HASO4.7H_2O$ (pH 7.4) and 1.5 mL of 2.0 M $NH_2OH.HCL$. The pH of the two-fold assay mixture was brought to pH 7.4 with 2.0 N NaOH before use. To 0.5 mL of the two-fold assay mixture, 0.4 mL of water and 0.1 mL of enzyme were added and incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 2.0 mL of ferric chloride reagent to the reaction mixture. The optical density was read at 540 nm and authentic γ-glutamyl hydroxamate was used as standard. Controls lacked sodium arsenate and ADP. Ferric chloride reagent was prepared by mixing 4.0 mL of 10% $FeCl_3.6H_20$, 1.0 mL of 24% Trichloroacetic acid, 0.5 mL of 6.0N HCl and 6.5 mL of water. Specific activity of GS is expressed as n mole γ- glutamyl hydroxamate formed per minute per mg protein.

Glutamate synthase (GOGAT; E.C.2.6.1.53) and NAD(P)H-Glutamate dehydrogenase (NAD(P)H - GDH; E.C. 1.4.1.4): Both enzymes were assayed using the same enzyme preparation. The enzymes GOGAT and NAD(P)H-GDH were assayed following the procedure of Thevanathan (1980). The reaction mixture for GOGAT contained 1.0 mL of 0.2 M potassium phosphate buffer (pH 7.0),).2 mL of 10.0 mM 2-oxoglutarate (neutralized with KOH, 0.2 mL of 1.0 mM NAD(P)H, 0.1 mL of enzyme and 4.0 mL of water. Glutamine (0.1 mL; 100 mM) was added 4 minutes after the addition of all the other reagents. The decrease in extinction at 340 nm was monitored. Σ_{340} NAD(P)H = 6.22 x 10^3 1 $mole^{-1}$ cm^{-1} is employed in calculating the amount of NAD(P)H oxidized. Activity of the enzyme is expressed as n mole NAD(P)H oxidized per minute per mg protein.

Activity of the enzyme was assayed following the procedure of Ahmad and Hellebust (1984) with some modifications. The reaction mixture in a final volume of 2.9 mL contained 1.5 mL of 0.1 M HEPES buffer (pH 7.8), 1.0 mL of glycine (75 µ moles), 0.1mL of ammonium acetate (300 µ moles) 0.1mL of 2-oxoglutarate (25 µ moles) and 0.1mL of NAD(P)H (0.25 µ moles). The reaction was initiated by the addition of 0.1 mL of dialyzed crude enzyme preparation and activity of the enzyme was determined by following the 2-oxoglutarate – dependant oxidation of NAD(P)H. The optical density was read at 340 nm. Results were presented as n moles NAD(P)H oxidized min^{-1} mg^{-1} protein at 30°C.

RESULTS

Nitrate assimilation by plants involves the participation of the enzymes of nitrate reduction and ammonia assimilation namely, nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). The effect of growing plants in soil amended with panchagavya preparations on these enzymes was studied by determining the levels or activities of the enzymes in the leaves of the experimental plants.

I Enzymes of nitrate reduction

a. *In vitro* Nitrate reductase (NR; E.C. 1.6.6.1)

In the control plants, the in vitro NR levels were always high in the leaves of *Oryza sativa* (413 n moles of NO_2 formed h^{-1} g^{-1} protein) as compared to other experimental plants. Among the pulses, *Vigna mungo* recorded the highest activity for the enzyme (311 n moles of NO_2 formed h^{-1} g^{-1} protein). The seedlings grown in soil amended with seaweed based panchagavya always registered higher activity for *in vitro* NR than their respective controls and those treated with traditional panchagavya as well (Figure 1).

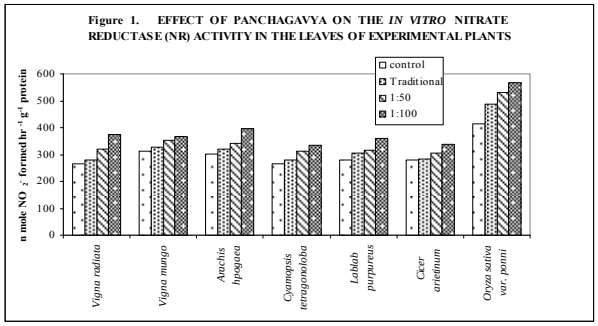


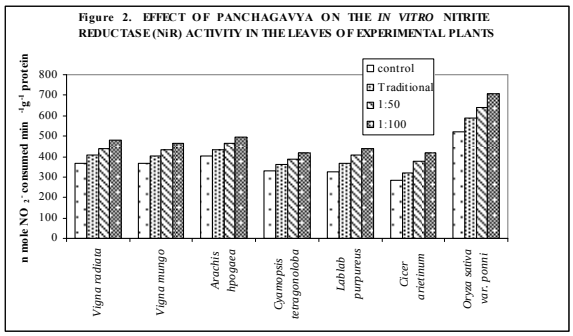
Figure 1. EFFECT OF PANCHAGAVYA ON THE *IN VITRO* NITRATE REDUCTASE (NR) ACTIVITY IN THE LEAVES OF EXPERIMENTAL PLANTS

Seaweed based panchagavya when used in low concentration (panchagavya: soil; 1: 100 v/v) recorded maximum activity for the enzyme in the leaves of all experimental plants. As compared to control, the quantum of increase in the *in vitro* NR levels in the leaves of these seedlings was in the range of 18 to 40%. Increasing the concentration of the seaweed based panchagavya in soil (panchagavya: soil; 1: 50 v/v) resulted in a decrease in the observed quantum of increase in NR levels as compared to other treatments. The seedlings grown in soil preparation amended with tradition panchagavya marginally increased the levels of the enzyme in all pulses. Nevertheless, the treatement was able to enhance the levels of the enzyme to appreciable quantities in rice.of increase in NR. However, the

seedlings grown in soil amended with seaweed based panchagavya had 17 to 32% more NR in their leaves in as compared to those grown in soil amended with traditional panchagavya.

b. *In Vitro* Nitrite reductase (NiR; E.C. 1.6.6.4)

Nitrite reductase levels in all the experimental plants were higher than that of Nitrate reductase (Figure 2). As observed for NR, panchagavya increased the *in vitro* NiR levels too in all the experimental plants. Similarly, the soil preparation with seaweed based panchagavya was more effective in enhancing the levels of NiR than the soil preparation with traditional panchagavya. Even at high concentration (1: 50; panchagavya: soil), appreciable levels of increase in the activity of NiR of leaves were evident in seedlings grown in soil amended with seaweed based panchagavya.

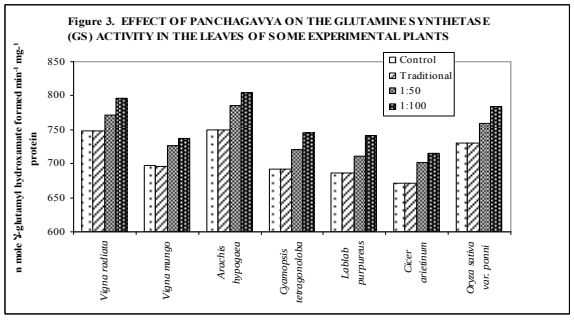


At this concentration, seaweed based panchagavya was able to increase NiR activity by 15 to 31% and at 1:100 dilution i.e. at low levels of panchagavya, the magnitude of increase was in the range of 22 to 47%. *Cicer arietinum* registered the maximum with 47% increase over its control (Fig. 10). Panchagavya in combination with seaweed extract was effectively promoting nitrate reduction in the experimental plants including rice.

II Enzymes of ammonia assimilation

a. Glutamine synthetase (GS; E.C. 6.3.1.2)

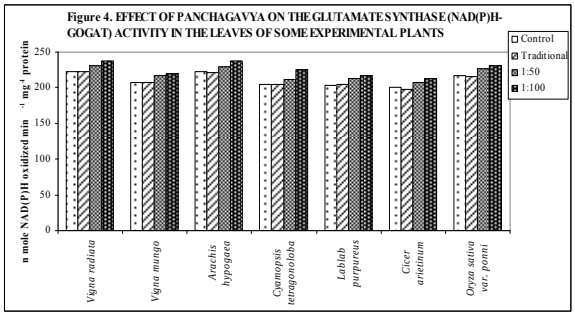
Glutamine synthetase levels were high in the legumes *Arachis hypogea* and *Vigna radiata* as compared to other experimental plants. Traditional panchagavya treatment either marginally decreased the levels of GS or had no effect (Figure 3). On the contrary, seaweed based panchagavya promoted GS activity in the leaves of experimental plants.



At 1: 100 dilution with soil, seaweed based panchagavya caused 7% increase in the levels of GS in *Arachis hypogea, Cyamopsis tetragonoloba, Lablab purpureus* and *Oryza sativa* as compared to both control and those grown in soil preparation amended with traditional panchagavya.

b. Glutamate synthase (NAD(P)H-GOGAT; E.C.2.6.1.53)

Initial GOGAT levels were more than 200 n moles min^{-1} mg^{-1} protein in all experimental plants (Figure 4). At both 1: 100 and 1:50 dilutions with soil, seaweed based panchagavya was able to marginally increase the levels of the GOGAT in the experimental plants. On the other hand, traditional panchagavya caused a slight decrease in GOGAT levels which is otherwise statistically significant in *Arachis hypogea, Cicer arietinum* and *Oryza sativa*.

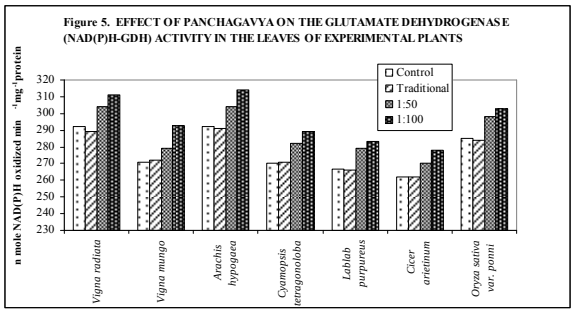


c. Glutmate dehydrogenase (NAD(P)H-GDH; E.C. 1.4.1.4)

NAD(P)H dependent GDH activity in the leaves of the experimental plants are shown in Figure 5.

Levels of NAD(P)H-GDH are nearly 40% less than that observed for GS. As observed for glutamate synthase, GDH also showed statistically significant decrease in its level in some experimental plants namely, *Vigna radiata, Arachis hypogea,*

Lablab purpureus and *Oryza sativa* when they were grown in soil amended with traditional panchagavya. Nevertheless, the enzyme recorded significant increase in its level in all experimental plants grown in seaweed based panchagavya irrespective of its concentration in soil. Percent increase in NAD(P)H-GDH levels in plants grown in soil amended with seaweed based panchagavya at 1: 100 dilution was in the range of 5 to 8%.



DISCUSSION

An investigation on the effect of panchagavya (both traditional and seaweed based) on the *in vitro* levels of the enzymes of nitrate assimilation namely, nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) the potential of panchagavya amended seaweed extract as a manure at low levels in enhancing the activities of these enzymes.

The *in vitro* levels of NR were always high in the leaves of seedlings grown in soil amended with panchagavya as compared to the control seedlings (Figure 1). The effect was more pronounced in seedlings grown in seaweed based panchagavya than those grown in traditional panchagavya registering high levels of the enzyme in the leaves of seedlings grown in soil preparations containing low levels of seaweed based panchagavya. The quantum of increase in the *in vitro* NR levels in the leaves of these seedlings was in the range of 18 – 40% (Figure 1). *In vitro* NiR levels too exhibited a similar response to treatment with panchagavya based soil preparations (Figure 2). The leaves of the experimental seedlings grown in soil preparations containing low quantities of seaweed based panchagavya had 22 – 47% more NiR than the leaves of the respective controls. Induction in the levels of NR and NiR in the experimental plants by panchagavya manure indicates its stimulatory effect on the nitrate reduction efficiency in these plants, when used at low quantities. An efficient nitrate reduction system would mean the generation of reduced nitrogen, ammonia in huge quantities and this in turn would warrant an efficient system of assimilation of the reduced nitrogen. The initial products of reduced nitrogen assimilation in plants have shown to be glutamine and glutamic acid (Aprison *et al.*, 1954; Leaf, 1959; Mulder *et al*., 1959, Jordan, 1960; Fowden, 1962; Kates and Jones, 1964; Grimes and Fottrell, 1966; Sims *et al*., 1968; Kretovich *et al*., 1970; Thevanathan, 1980; Bhavani, 1983; Gangadharan, 1995; Veerabadran, 1995). The enzymes glutamine synthetase (GS) (Thevanathan, 1980; Bhavani,1983; Subramanian and Rajalakshmi, 1989; Gracia Fernandez *et al*., 1994;), glutamate synthase (GOGAT) (Thevanathan, 1980 ; Bhavani, 1983; Galvan *et al*., 1984; Fischer and Klein, 1988; Zehr and Falkowshi, 1988;) and glutamate dehydrogenase (GDH) (Kretovich *et al*., 1970; Brown *et al*., 1974; Gayler and Morgan, 1976) have been shown to be the key enzymes involved in the primary assimilation of ammonia involved in both lower and higher plants. These enzymes have been detected in the experimental plants in sufficient levels to account for the observed nitrate and nitrite reductases (Figures 3, 4 and 5). Unlike that observed for NR and NiR, traditional panchagavya manure did not induce the levels of GS in the experimental seedlings and in fact decreased the levels of GS in *Vigna radiata*, *Cicer arietinum* and *Oryza sativa* var. *ponni* (Figure 3). Nevertheless, seaweed based panchagavya manure was able to cause a clear induction in the levels of GS to an extent of 5 – 7%. A concomitant increase in the levels of GOGAT (NAD(P)H – dependant) could be observed in leaves of the experimental plants grown in seaweed based panchagavya manure only. Traditional panchagavya manure either reduced the levels of both glutamate synthase and glutamate dehydrogenase or had any effect on these enzymes. Nevertheless, seaweed based panchagavya manure could induce an increase in the levels of NAD(P)H-GDH too (Figure 5). These results indicate that seaweed based panchagavya can be used as a manure at very low concentration (1:100 v/v; manure: soil) rather than traditional panchagavya. The positive stimulatory effect on NR and NiR in the experimental plants and the negative effect on the enzymes of ammonia assimilation i.e. GS, GOGAT and GDH of traditional panchagavya manure warrants further studies to account for its discrimination against the enzymes of ammonia assimilation.

CONCLUSIONS

Seaweed based panchagavya exhibited a promotory effect on the enzymes of nitrate reduction

i.e. NR and NiR and the ammonia assimilation i.e. GS, NAD(P)H-GOGAT and NAD(P)H-GDH in the experimental pulses and rice, when used as a manure in the soil preparation at a dilution of 1 : 50 and 1 : 100 (seaweed panchagavya : soil). Traditional panchagavya exhibited a negative effect on the enzymes of ammonia assimilation in these plants.

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