Journal of Pharmaceutical and Scientific Innovation

www.jpsionline.com (ISSN : 2277 -4572)

Research Article

KINETIN FUNCTIONS IN NITRATE ASSIMILATION IN LEAVES OF ARSENIC STRESSED *VIGNA RADIATA*

Govinda [1], Asha Sharma [2], Anju Dhiman [3]*, Sandeep Singh [1], Nidhi [1]
[1]Department of Biochemistry, Maharshi Dayanand University, Rohtak, Haryana, India
[2]Department of Botany, MaharshiDayanand University, Rohtak, Haryana, India
[3]Department of Pharmaceutical Sciences, MaharshiDayanand University, Rohtak, Haryana, India
ad_mdu@rediffmail.com

DOI: 10.7897/2277-4572.06144

Received on: 27/12/16 Revised on: 16/02/17 Accepted on: 25/2/17

ABSTRACT

The toxic effects of arsenic were studied in leaves of arsenic V*igna radiata*(cv) were examined. The hydroponic cultivation of *Vigna radiata* in medium (50µM) and high Arsenic (As) (100µM) stress and low (2µM) and high (5µM) kinetin levels with their corresponding combinations of both were supplied to the plant in Hoagland's solution. The evaluation of overall growth and biomass accumulation in arsenic stress and kinetin treatment were done during the study. The physical parameters (fresh/dry weights root and shoot lengths) were monitored. The analysis of behaviour of nitrate assimilating enzymes in leaves of *Vigna radiate* in the presence of medium and high content of arsenic and kinetin treatments were executed. The investigation of modulation of nitrate and ammonium accumulation in as stressed *Vigna radiata* leaves by kinetin were recorded. The activity of nitrate reductase was inhibited by 100µM As while effect of 50µM As was insignificant. The grades showed significant accumulation of total nitrate in arsenic (50 µM and 100 µM) and kinetin (2 µM and 5 µM) doses as compared to control. The combination of arsenic and kinetin further augmented the total nitrate content notably as compare to arsenic treated plants (50 µM and 100µM).

KEY WORDS: Arsenic, nitrate reductase, nitrite reductase, physical parameters, *V. radiata.*

INTRODUCTION

Arsenic (As) is referred to as a metal, and in the context of toxicology, as a heavy metal. Since most arsenic compounds lack colour or smell, the presence of arsenic is not immediately obvious in food, water or air, thus presenting a serious human health hazard given the toxic nature of the element. Indeed, the very name arsenic is synonymous with poison, since the consequence of its long and nefarious history[1]. The major route of chronic-exposure for As is through consumption of contaminated drinking water. As exposure has been shown to result in marked elevation in reactive oxygen species (ROS), lipid peroxides along with decrease in superoxide dismutase and reduced glutathione level causing oxidative DNA damage, severe pathological changes and even apoptosis in neural cells, thus causing brain damage[2]. Mitochondria play central role in energy metabolism[3] and are the major sites of ROS production as by-products of the electron transport chain (ETC)[4]. The oxidative stress leads to alterations in mitochondrial DNA, lipids and proteins due to peroxidative damage of amino acids, carbohydrates, lipids, proteins and nucleic acids[5]. The toxic effects of As on biological systems have been reported by several authors but the mechanism of As toxicity is not yet completely understood. Some important toxic effects of As on plants include interference in uptake of macronutrients, inhibition of nitrate As assimilation[6], decline in nitrogen fixation, reduction in ATPase activity[7] and disturbance of chlorophyll synthesis and CO_2 fixation[8]. The activities of nitrate assimilating enzymes have been analyzed in a number of As stressed plants. The nitrate reductase has been found to decrease in response to As stress in *Oryza sativa*[9], *P. vitata*[10], *Triticum aestivum*[11], *Cicer arietinum*[12] etc. The nitrite reductase has also been found to decreased in *Oryza sativa*[9], *P. vitata*[10], *Triticum aestivum*[11].The glutamine synthetase has also been found to be decreased in *Triticum aestivum*[11], *Oryza sativa*[9]and*P. vitata*[10]. The glutamate synthase has also been found to decrease in *Triticum aestivum*[11]. Kinetin is a type of cytokinin which is a plant hormone that plays a major role in cell division and cell differentiation. Kinetin stimulates an increased composition of haploid ascospores in *Saccharomyces cerevisiae*[13].

MATERIALS AND METHODS

Source of biological material

Seeds of *Vigna radiata*(SML-660) were purchased from local market, Rohtak.

Growth and collection of plant material

Seeds of *Vignaradiata*were soaked overnight in minimum distilled water (DW) and surface sterilized next day in 0.1% $HgCl_2$. The pre-soaked and sterilized seeds were then regerminated hydroponically in nutrient solution containing 8mM KNO_3, 2mM $Ca(NO_3)_2$, 1mM KH_2PO_4, 1mM $MgSO_4$ and micronutrients: 30µM H_3BO_4, 5µM $MnSO_4$, 1µM $CuSO_4$, 1µM $(NH_4)_6Mo_7O_{24}$ and 1µM $ZnSO_4$[14]. After 3 days of normal Hoagland treatment, the solutions with 2µM and 5µM kinetin were used to irrigate two sets of plants besides a third set of plants without kinetin as control. Two more sets of plants were treated with 50µM and 100µM As containing nutrient solutions. Final two sets of plants were treated with 50µM and 100µM As added to 2µM and 5µM kinetin respectively. The

seedlings were collected for leaves from all the seven sets after 5 days of treatment (Figure 1).

Physical parameters

The shoot length and fresh weight were measured from 6 randomly selected plants from each treatment and kept in oven at 70°C for one day for dry weight measurement.

Assessment of enzymes

***Nitrate reductase (NR)*:** The NR was estimated as actual NR activity by the method of *Dguimiet al* (2009)[14]. The assay mixture contained 1.2 ml of 0.1M potassium phosphate buffer (pH 7.5, containing 10mM $MgCl_2$), 0.2ml 0.2M KNO_3 and 0.2 ml of 4.0mM NADH. The reaction was initiated, after 2 min preincubation at 30◦C, by adding 0.4 ml of leaf extract and conducted incubation for 30 min. The blank contained 0.3ml DW in place of leaf extract. After incubation for 30min, 1ml of 1% (w/v) sulfanilamide in 3M HCl (*Griess Reagent 1*) and 1ml of 0.02% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride (*Griess Reagent 2*) were added to develop pink colorwhose absorbance was noted at 540nm. One unit of NR activity was defined as 1 µg NO_2^-formed per hour. For standard curve, 10 mg sodium nitrite was dissolved in 100ml DW and diluted to 10 times for 10µg/ml stock. From this stock, 0.4ml each of 0.03-0.14µmoles/ml nitrite solutions was taken in reaction mixture in place of sample for standard curve.

Glutamine Synthetase (GS)

GS activity was estimated by the modified method of Wang *et al.,*2008[15]. The reaction mixture consisted of 1.6 ml of 0.1M Tris–HCl buffer (pH 7.5) with 20mM sodium glutamate, 80mM $MgCl_2$, 20mM sodium arsenate and 20mM NH_2OH. The reaction was started by addition of 0.5ml 0.75mM ATP and 0.4 ml enzyme extract, incubated for 1 h at 37°C, and stopped by adding 1ml of ferric chloride reagent (equimolar mixture of 0.37M $FeCl_3$, 0.2M trichloroacetic acid and 0.6M HCl). After centrifugation (5000*g* for 15 min at 4°C), absorbance of the supernatant was read at 535 nm. One unit of GS activity was expressed as 1µmol ɣ-glutamylhydroxamate formed per minute. Calculation was done from extinction coefficient of ferric hydroxamate ($\gamma = 0.25\ mM^{-1}\ cm^{-1}$).

***Glutamate synthase* (GOGAT)**

It was estimated spectrophotometrically by monitoring oxidation of NADH (ε=6.22 $mM^{-1}\ cm^{-1}$) at 340nm [16]. The enzyme catalyzes conversion of a molecule each of 2-oxoglutarate and glutamine into two molecules of glutamate while utilizing an NADH in process.2ml assay mixture[17]was 1.9ml 0.1M potassium phosphate buffer (pH 7.5) containing 12mM 2-oxoglutaric acid, 12mM glutamine, 0.15mM NADH and 0.1 ml enzyme extract. The activity was expressed in units with 1 unit representing the enzyme catalyzing 1nmol NADH oxidation per minute.

Estimation of total ammonium

It was estimated by the method of El-Shora and Ali (2011) using Berthelot reaction[18]. The samples used were dried leaf samples crushed in 8 ml DW and filtered with Whatmann filter paper. For reaction, 1ml plant sample was added to 1 ml phenol reagent (0.005% sodium nitroprusside in 1% phenol). 1ml alkaline sodium hypochlorite (0.042% sodium hypochlorite in 0.5% NaOH) was added and incubation was done at 37°C for 20min. Absorbance was noted at 625nm against blank having DW in place of plant extract. The total ammonium was calculated against standard curve of ammonium chloride (**Fig. 5**). For this, 53.49mg ammonium chloride was dissolved in 100ml DW for 10µmoles/ml stock and 0.1ml of concentrations in the range 2-10µmoles/ml were taken in reaction mixture in place of sample.

Estimation of nitrate

Nitrate concentration in the dried samples was determined according to *Xiong et al*., (2006) with slight modifications[19]. In this method, salicylic acid reacts with nitrate in acidic conditions to form nitrosalicylic acid having maximum absorbance at 410 nm. Dried tissues were powdered in 8ml DW after noting their weights. The samples were shaken and filtered through Whatman filter paper. Nitrate in the filtered solution was determined by adding a 0.4ml. sample aliquot to 2.6ml of 1% (w/v) salicylic acid in conc.H_2SO_4. The mixture was cooled at room temperature and developing color was measured at 410nm against blank having 0.4ml DW in place of sample. Calculation was done from the standard curve of 20-100µg/ml nitrate.

RESULTS

Arsenic stress may be mitigated by kinetin- a type of cytokinin which is a plant hormone that plays a major role in cell division and cell differentiation. In 1955, Miller and Skoogisolated kinetin as a compound from autoclaved herring sperm DNA which had cell division promoting actions. Kinetin promotes an increased composition of haploid ascospores in *Saccharomyces cerevisiae*[13]. Kinetin inhibits auxin induced rooting of mung bean cuttings effectively as the intercalating DNA chemicals proflavine, acridine and ethidium bromide[20].

Physical growth assessments

The treatment of plants with 50 and 100 µMAs and 2 µM and 5 µM kinetin as well as their combinations did not yield any significant changes in any of the three physical parameters studied (Table 1).

Table 1: Effect of nickel treatment on shoot length, fresh masses and dry masses of leaves of *Vigna radiata* plant.

Treatment	Fresh weight(g) [mean ± SD]*	Shoot length(cm) [mean ± SD]*	Dry Weight (mg) [mean±SD]
Control	0.231±0.050	17.075±1.93	16.15±3.04
50µM As	0.250±0.054	19.62±2.414	17.61±5.27
100µM As	0.198±0.042	16.11±2.28	18.86±3.042
2µM kinetin	0.302±0.024	19.33±1.967	14.61±4.36
5µM Kinetin	0.220±0.029	16.63±1.862	16.88±4.56
50µM As + 2µM Kinetin	0.259±0.067	17.26±2.88	17.74±3.72
100µM As + 5µM Kinetin	0.195±0.046	16.94±2.56	15.77±6.02

Assays of Enzymes

***Nitrate reductase*:** The activity of nitrate reductase was inhibited by 100µM As while effect of 50µM As was insignificant. The activity of this enzyme was stimulated by 5µM kinetin while effect of 2µM kinetin is insignificant (Figure 1). The stimulating effect of 5µM kinetin was strong even in the presence of 100µM As. The effect of kinetin may probably be due to an increase in nitrate uptake and stimulation of nitrate reductase by nitrate[21]. As mediated inhibition of nitrate reductase has also been reported in*Oryza sativa*[9], *P. vitata*[10], *Triticum aestivum*[11]and *Cicer arietinum*[12].

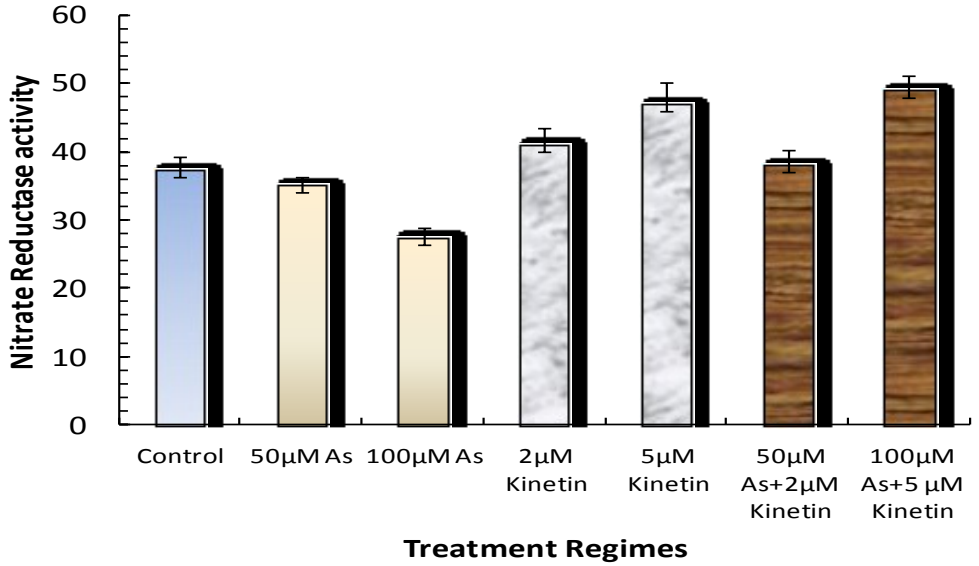

Figure 1: Changes in nitrate reductase activity (µmoles nitrate /min/g fw) of *Vigna radiata*seedlings under various regimes of As and kinetin [p*<0.05(probably significant), $p < 0.02 (Definitely significant), #p<0.01 (Highly significant), ##p<0.01 (highly significant from 100µM As)]**

***Nitrite reductase*:** The activity of nitrite reductase was not significantly affected by 50µM and 100µM As but the higher concentration of kinetin (5µM kinetin) stimulated the same. When lower concentration of both As and kinetin (50µM As+2µM kinetin) was given, there was no significant change in nitrite reductase as compared to control (Figure 2). The higher concentration of both combination of As and kinetin (100µM As+5µM kinetin) showed an elevation of nitrite reductase activity as compared to control as well as 100µM As stressed plants. Hence, 5µM kinetin was found to be a strong stimulator for nitrite reductase in arsenic stressed plants taken for the study. As mediated inhibition of nitrite reductase has been reported in *Oryza sativa*[9], *P. vitata*[10]and *Triticum aestivum*[11].

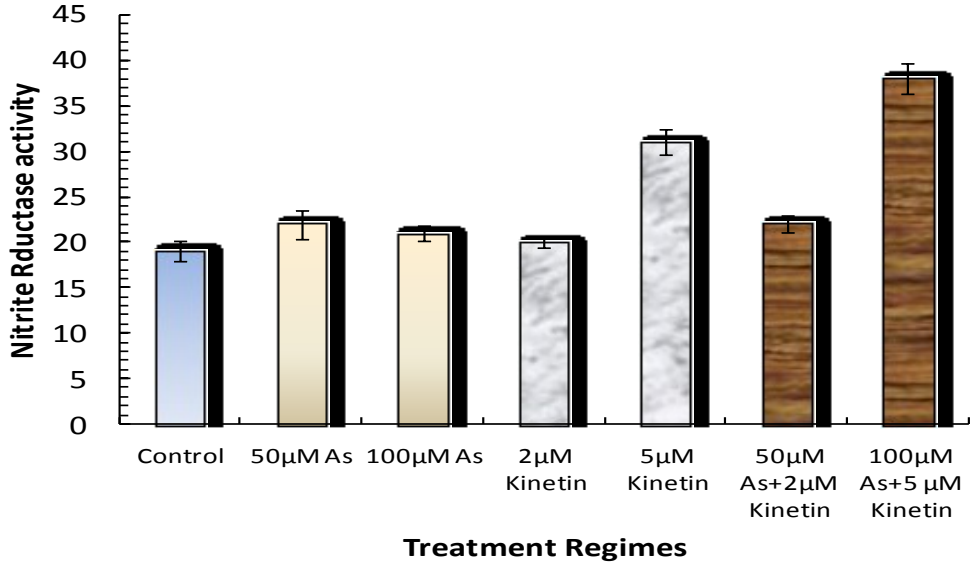

Figure 2: Changes in nitrite reductase activity (µmoles nitrite /min/g fw) of *Vigna radiata* seedlings under various regimes of As and kinetin [p*<0.05(probably significant), $p < 0.02 (Definitely significant), #p<0.01 (Highly significant), ##p<0.01 (highly significant from 100µM As),]**

***NADH-GOGAT*:** The activity of glutamate synthase was shown to be decreasedunderAs stress when compared with control, however, 5µM kinetin stimulated this enzyme (Figure 3). The kinetin dominated the metal stress too. The higher metal concentration along with kinetin increased glutamate synthase activity as compared to control as well as only metal treated plants. The lower kinetin (2µM kinetin) also increased GOGAT activity of lower metal concentration (50µM As) at par with control plants. As mediated inhibition of glutamate synthase has been reported in *Triticum aestivum* (Ghosh*et al.,* 2013), *Oryza sativa* (Jha*et al*., 2004) and *P. vitata* (Singh *et al*., 2009).

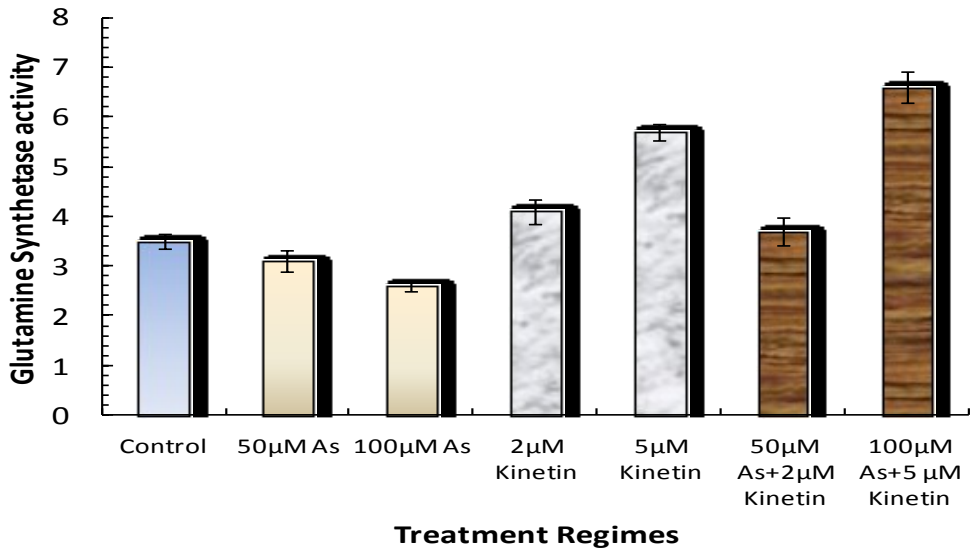

Figure 3: Changes in glutamine synthetase activity (µmoles ɣ-glutamylhydroxamate formed/ min/g fw) of *Vignaradiata*seedlings under various regimes of As and kinetin [*p<0.05 (probably significant), $^{\$}$p < 0.02 (Definitely significant), $^{\#}$p<0.01 (Highly significant), $^{\#\#}$p<0.01 (Highly significant from 100µM As)]

Estimation of nitrate

To check whether As, kinetin or their co-treatment affect the nitrogen metabolism, total nitrate content was also estimated in treated and control plants. The results showed significant accumulation of total nitrate in As (50 µM and 100 µM) and kinetin (2 µM and 5 µM) treatments as compared to control. Co-treatment of As and kinetin further increased the total nitrate content significantly as compare to As treated plants (50 µM and 100µM). So, kinetin was very strong stimulant of nitrate uptake (Figure 4). This increased uptake of nitrate was probably responsible for increase in nitrate reductase activity as already shown.

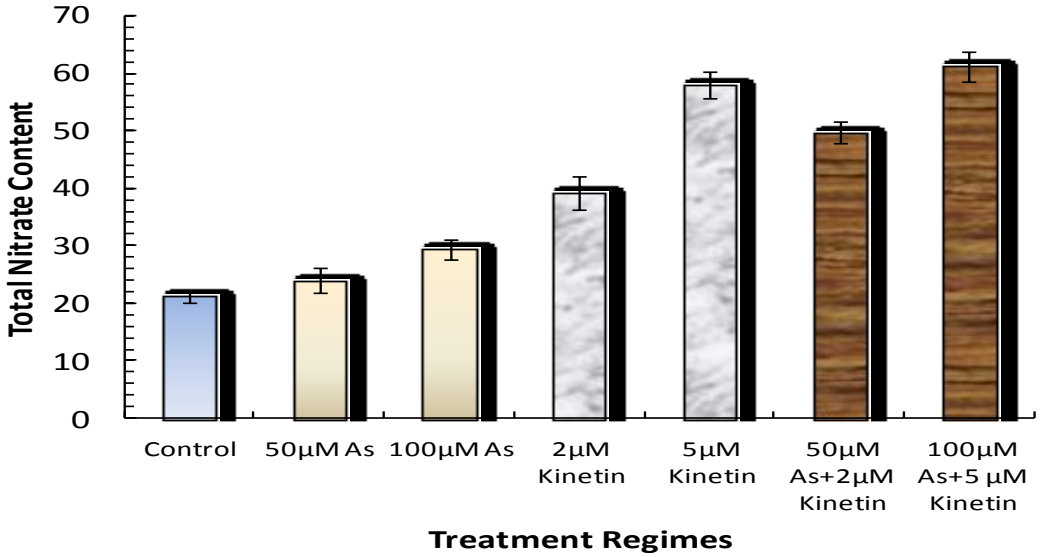

Figure 4: Changes in nitrate content (µg/g fw) of *Vignaradiata*seedlings under various regimes of As and kinetin [$^{\$}$p<0.02 (Definitely significant), #p<0.01 (Highly significant), $^{\%\%}$p<0.01 (Highly significant from 50µM As), $^{\#\#}$p<0.01 (Highly significant from 100µM As)]

Estimation of Ammonium

To check whether As, kinetin or their co-treatment affect the nitrogen metabolism total nitrate content was estimated in treated and control plants. The results showed significant accumulation of total nitrate in 100 µM As and kinetin (2 µM and 5 µM) treatments as compared to control (Figure 5). Co-treatment of As and kinetin in lower combination increased the total nitrate content significantly as compare to control as well As treated plants (50 µM and 100µM) but higher combination maintained ammonium content at 100 µM kinetin level. Accumulation of ammonium in kinetin treated plants is probably due to stimulation of GS and GOGAT which was also done by kinetin (shown earlier).

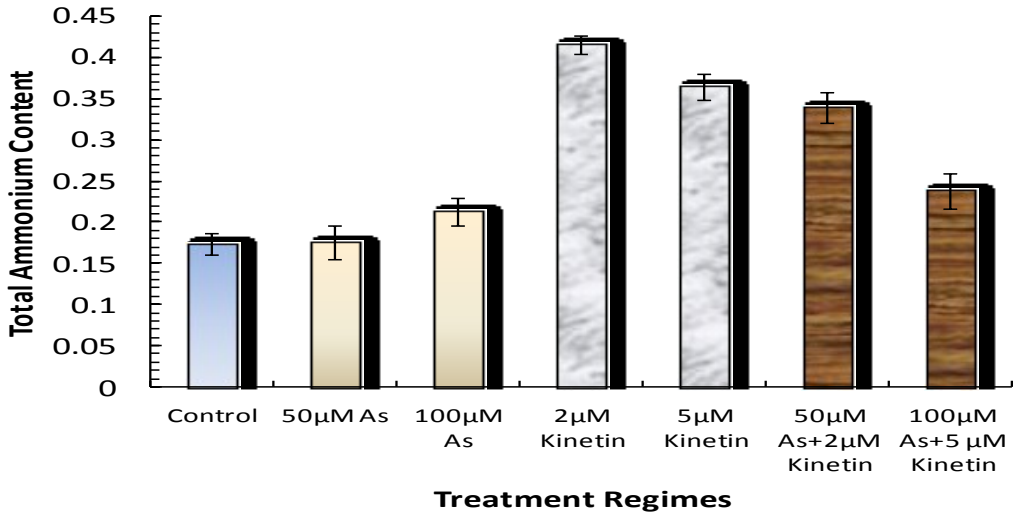

Figure 5: Changes in ammonium content (µmoles/g fw) of *Vigna radiate* seedlings under various regimes of As and kinetin [p*<0.05(probably significant), #p<0.01 (Highly significant), %%p<0.01 (Highly significant from 50µM As)]**

DISCUSSION

Arsenic is known to interfere in uptake of macronutrients, inhibition of nitrate Arsenic assimilation, decline in nitrogen fixation, reduction in ATPase activity and disturbance of chlorophyll synthesis and CO_2 fixation. The plant taken up was *Vigna radiata*- the main pulse of India and kinetin (2µM and 5µM), arsenic (50µM and 100µM) and their combinations were given for five days. The biomass production did not show any significant changes in kinetin and arsenic treatments, there were no changes in fresh/dry weights of shoots also. The kinetin showed some novel effects in the plant system on the nitrate reductase, nitrite reductase and other parameters. The greater arsenic (100µM Arsenic) had very significant role in decreasing the activity of the nitrate reductase. But the kinetin at high level (5µM) supported the NR activity. The combination of kinetin with the arsenic in high level also favoured increase in the NR activity and in this way, kinetin dominated the salt stress. Nitrite reductase was unresponsive to arsenic stress but arsenic at 5µM still further increased its activity alone as well as in combination with higher arsenic level. But, the activity of glutamine synthetase was reduced strongly by high As level (100µM) while kinetin at both studied levels stimulated its activity greatly. The response of NADH-GOGAT was also very similar to that of glutamine synthetase.

CONCLUSION

It was concluded from the study that higher arsenic (100µM Arsenic) had very noteworthy role in declining the activity of the nitrate reductase. But the kinetin at elevated level (5µM) supported the NR activity. The combination of kinetin with the arsenic in high level also favoured increase in the NR activity and in this way, kinetin dominated the salt stress. Nitrite reductase was unresponsive to arsenic stress but arsenic at 5µM still further increased its activity alone as well as in combination with higher arsenic level. But, the activity of glutamine synthetase was reduced strappingly by high arsenic rank (100µM) while kinetin at both studied levels stimulated its activity significantly. The response of NADH-GOGAT was also very similar to that of glutamine synthetase.

REFERENCES

1. Mandal BK, Suzuki KT. Arsenic around the world: a review. *Talanta* 2002; 58: 201-235.
2. Dwivedi N, Flora SJ. Concomitant exposure to arsenic and organophosphates on tissue oxidative stress in rats. *Food Chem Toxicol* 2011; 49: 1152-1159.
3. Pelicano H, Feng L, Zhou Y, Carew JS, Hileman EO, Plunkett W. Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis inhuman leukemia cells by a reactive oxygen species mediated mechanism. *J Biol Chem* 2003; 278: 37832–37839.
4. Batandier C, Fontaine E, Keriel C, Leverve XM. Determination of mitochondrial reactive oxygen species: methodological aspects. *J. Cell Mol. Med* 2002; 6: 175-187.
5. Bellomo G, Orrenius S. Altered thiol and calcium homeostasis in oxidative ehepatocellular injury. *Hepatology* 1985; 5: 876-882.
6. Hernandez LE, Carpena-Ruiz R, Garate A. Alterations in the mineral nutrition of pea seedlings exposed to heavy metal. *J Plant Nutr* 1996; 19: 1581-1598.
7. Fodor E, Szabo-Nagy A, Erdei L. The effects of heavy metal on the fluidity and H^+-ATPase activity of plasma membrane from sunflower and wheat roots. *J Plant Physiol* 1995; 147: 87–92.
8. De Filippis LF, Ziegler H. Effect of sublethal concentrations of heavy metal on the photosynthetic carbon

reduction cycle of *Euglena*. *J Plant Physiol* 1993; 142: 167-172.
9. Jha AB, Dubey RS. Arsenic exposure alters activity behaviour of key nitrogen assimilatory enzymes in growing rice plants. *Plant Growth Reg* 2004; 43: 259–268.
10. Singh N, Maa LQ, Vu JC, Raj A. Effects of arsenic on nitrate metabolism in arsenic hyperaccumulating and non-hyperaccumulating ferns. *Environ Pollut* 2004; 157: 2300–2305.
11. Ghosh S, Saha J, Biswas AK. Interactive influence of arsenate and selenate on growth and nitrogen metabolism in wheat seedling. *Acta Physiol* 2014; 35: 1873-1885.
12. Kumar S, Kumar S, Prakash P, Singh M. Antioxidant defense mechanism in Chickpea Cu and As toxicity. *Int. J. Plant Physio. Biochem.* 2014; 6: 40-43.
13. Laten HM. Cytokinins affect spore formation but not cell division in the yeast *Saccharomyces cerevisiae*. *Biochimicaet Biophysica Acta (BBA)-Molecular Cell Res* 1995; *1266*: 45-49.
14. Dguimi HM, Debouba M, Ghorbel MH, Gouia H. Tissue-specific cadmium accumulation and its effects on nitrogen metabolism in tobacco (*Nicotiana tabaccum, Bureley v*. Fb9). *CR Biologies* 2009; 332: 58-68.
15. Wang L, Zhou Q, Ding L, Sun Y. Effect of cadmium toxicity on nitrogen metabolism in leaves of *Solanum nigrum* L. as a newly found cadmium hyperaccu-mulator. *J. Haz Mat* 2008; 154: 818-825.
16. Gajewska E, Sklodowska M. Nickel-induced changes in nitrogen metabolism in wheat shoots. *J Plant Physiol* 2009; 166: 1034-1044.
17. Rajasekaran C, Kalaivani T, Jayakumararaj R, Singh A, Pusalkar VR and Marimuthu R. Impact of altitudinal gradient on ammonium assimi-latory enzymes in *Rauvolfia teraphylla* L. (Apocyanaceae) – A perennial medicinal herb. *Ethnobot Leafl* 2009; 13: 413-421.
18. El-Shora HE, Ali AS. Changes in activities of nitrogen metabolism enzymes in cadmium stressed marrow seedlings. *Asian J. Plant Sci*. 2011. DOI: 10.3923/ajps.2011, pp. 1-8.
19. Xiong ZT, Liu C, Geng B. Phytotoxic effects of copper on nitrogen metabolism and plant growth in *Brassica pekinensis* Rupr. *Ecotoxicol. Environ Saf*. 2006; 64: 273–280.
20. Chen J, Witham FH, Heuser CW. Inhibition of NAA-induced adventitious roots in mung bean cuttings by kinetin, zeatin, ethidium bromide and other DNA intercalators. *World Wide Web J Biol* 1996; 1:1-8.
21. Garg N, Singla R. Nitrate reductase activity in roots and leaves of chickpea cultivars under salt stress. *Spanish J Agric Res* 2005; 3: 248-252.

How to cite this article:
Govinda *et al*. kinetin functions in nitrate assimilation in leaves of arsenic stressed *Vigna radiata*. J Pharm Sci Innov. 2017;6(1): 8-13.

Source of support: Nil, Conflict of interest: None Declared