RESEARCH ARTICLE

INTERACTIVE EFFECTS OF VANADIUM AND PHOSPHORUS ON THEIR UPTAKE, GROWTH AND HEAT SHOCK PROTEINS IN CHICKPEA GENOTYPES UNDER HYDROPONIC CONDITIONS

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Abstract

The present study was carried out to examine the interaction of vanadium and phosphorus and changes in heat shock genes to optimize the growth of chickpea genotypes. Two sets of hydroponic experiments were carried out using vanadium and phosphorus with five-level central composite design. Five levels of vanadium (0-1180 µM) and phosphorus (0-1000 µM) were used to evaluate their interactive effects. Plants fresh biomass and uptake of vanadium and phosphorus were influenced by vanadium and phosphorus application. Enhanced fresh biomass was most likely a result of increased phosphorus uptake by chickpea genotypes. Addition of vanadium induced toxic effects while, higher concentration of phosphorus alleviated its toxic effects. The obtained results also indicated that lower vanadium concentration promoted phosphorus absorption however; higher concentration of vanadium inhibited the phosphorus uptake. The morphological changes in leaves indicated that the cells were deformed and reduced in size when treated with higher vanadium levels with fixed phosphorus while, there was little deformation and reduction in cells size were observed when plants were treated with higher levels of phosphorus with fixed vanadium. Whereas, the proportion of deformation of cells were higher in Balkasar as compared to C-44 genotype. The results also showed that at elevated vanadium with fixed phosphorus, Hsp70 was expressed only in C-44 while, not in Balkasar however, Hsp90 and GAPDH showed non-significant results.

Keywords: *Cicer arietinum* L.; Vanadium; Phosphorus; Leaf morphology; Heat shock proteins; Biomass

1. Introduction

Over the last few decades, many countries have experienced rapid increase in economic development due to industrialization and urbanization which has also enhanced the heavy metals demand and consequently increased the anthropogenic environmental emissions. In particular, soils pollution with variety of heavy metals including vanadium has become serious issue worldwide and affecting agricultural productivity, livestock, and human beings (Imtiaz et al., 2015a). Vanadium is a ubiquitous trace metal in the atmosphere and widely distributed and naturally occurred in soil. Its average amount in Earth crust is similar to zinc and more common than copper or nickel (Perron, 2001; Moskalyk and Alfantazi, 2003). Vanadium is the 22nd most frequent and 5th most abundant element among all the transitional metals with an average of 10-220 mg kg^{-1} in the Earth crust (Sachin et al., 2011). However, the soils which are directly under the use of human beings contain much higher amount of vanadium (1,510 to 3,600 mg kg^{-1}) such as mining areas contain vanadium up to 738 mg kg^{-1} and 3505 mg kg^{-1} (Panichev et al., 2006; Teng et al., 2006).

There are different ways through which vanadium could enter into the environment such as weathering, combustion, atmospheric deposition, anthropogenic activities, and so on. Previous studies provide strong evidence for significant increases of vanadium into the environment in recent decades (Mejia et al., 2007). Additionally, vanadium has been put in the same class as Hg, Pb and As, because of its potentially dangerous effects (Naeem et al., 2007).

Recently, vanadium pollution is becoming major environmental concern worldwide, it occurs naturally in the soils and its higher concentration impairs plants growth and productivity (Imtiaz et al., 2015a; Imtiaz et al., 2015b). At present, the effects of vanadium on genetics, its distribution among the living organisms and toxicity have become the topic of research. Previously reported that the bioavailability of vanadium by plants is highly toxic and depend upon its chemical forms. Among all the chemical forms of vanadium, vanadate induced toxic effects on growth, gene expression, alter cellular functions and other physiological and chemical phenomena and higher concentrations of vanadium caused constrain plants growth and development (Anke, 2004; Olness et al., 2005; Marcano et al., 2006; Xi-yuan et al., 2012; Imtiaz et al., 2015b). Previous studies also indicated that vanadium interacts with protein, protein-DNA interactions, and imbalance the enzymatic activities (Anke, 2004; Goc, 2006). There are reports which reveal the direct effects of vanadium on plant growth and ultimately damage food quality

(Vaccarino et al., 1983), and there are all possibilities of this being repeated in future.

Moreover, the interaction between vanadium and phosphorus required more attention because both are chemically analogues (Ding et al., 1994; Rehder, 2003). Therefore, vanadate and phosphate competes each other for sorption on soils sites however, this competition increase the vanadium concentrations in solution due to reduction process. Moreover it is confirmed that vanadium inhibits and impairs the phosphate metabolizing enzymes like phosphatases, lyases, synthases, ATPases etc. (Ding et al., 1994; Stankiewicz et al., 1995; Olness et al., 2001). The vanadate interferes during metabolic processes involving phosphate in cytoplasm of the cell (Bowman, 1983). Vanadium is a potent enzymes inhibitor and can be used to characterize the enzymes involving in phosphate transfer (Olness et al., 2001).

Phosphorus is listed as second most critical and essential nutrient for plants because of its imperative role in metabolic activities. Previous studies well documented about the role of phosphorus to increase the growth as well as yield of plants (Saini et al., 2004; Gan et al., 2016). Ae et al. (1991) reported that chickpea plants are more efficient for phosphorus acquisition from soil because their roots secrete acid exudates which solublize the phosphorus compounds. Mainly, the plants absorb phosphorus through mass flow and diffusion processes (Saini et al., 2004; Singh et al., 2010). The plants absorb phosphorus as inorganic-phosphorus (Pi) however, plants exhibited both: high and low affinity mechanisms to transport inorganic-phosphorus across the plasma membrane. After absorption, inorganic-phosphorus transported within the plants via PHT1 proteins (Preuss et al., 2010, 211). The change in pH significantly influences the phosphorus uptake by plants such as at pH <6.0, mostly $H_2PO_4^-$ species found and at higher pH, HPO_4^{2-} found and plants mostly absorb phosphorus as $H_2PO_4^-$ (Furihata et al., 1992). Previous studies also have confirmed that application of phosphorus at early stage is necessary for the development of roots including early and secondary roots and root hairs, leaf development and tillering, for flowering, for grain formation, for higher yield and for nodules formation in chickpea plants (Meena et al., 2002; Jat and Ahlawat, 2006; Singh et al., 2010).

Vanadium uptake by plants depends on various environmental factors like soil type, nutrient supply, and medium pH. Therefore, elucidation of the relationship between vanadium and phosphate is important for better understanding and efficient crop production under polluted conditions. Moreover, the interaction between phosphate and vanadium needs careful attention because vanadium had the ability to reduce easily than phosphorus moreover; vanadate and

phosphate are chemical analogues and vanadium compounds (oxyaanion/oxonion) can replace the phosphate in nutrient pool (Ding et al., 1994; Stankiewicz et al., 1995; Olness et al., 2001). Therefore, vanadium potentially affects the enzyme reactions in which phosphate is a major component. Previous studies also confirmed that vanadate uptake by the plants is mediated by the phosphate carriers in roots (Seargeant and Stinson, 1979; Ding et al., 1994).

Little is known about the interactive effects between vanadium and phosphate on plants growth and uptake of vanadium and phosphate. The hypothesis of the present study was that two factors vanadium, phosphate and their different combinations in the growth media could influence the plant growth (biomass), phosphorus uptake and vanadium accumulation of chickpea genotypes. Therefore, the present study was aimed with the following objectives: (1) to determine the interactive effects and optimize the combination of vanadium and phosphorus for the growth chickpea genotypes, and (2) to determine the biochemical changes against interactive effects of vanadium and phosphorus.

2. Materials and Methods

2.1. Experimental setup

Two sets of hydroponic experiments (Table 1 and 2) were carried out in the present study to determine the interactive effects of vanadium and phosphorus in chickpea plants. The treatments were consisted on two factors: vanadium and phosphorus, five levels of each factor were taken. The treatments levels of both sets of experiment were determined according to the codes levels of two-factors with five levels central composite design (Box et al., 1978). The relationship between coded factors (x) and real values of treatment (X) was found as: $x = (X - X_0)/ \Delta j$, where X_0 stand for zero level. The upper values of vanadium and phosphorus were 1180 and 1000 µmol, respectively, while the lower values for both vanadium and phosphorus were 0 µmol. The zero (X_0) and scaling factor/change interval (Δj) of vanadium and phosphorus were computed according to their respectively highest and lowest levels with the help of following formulas:

$$x = (X - X_0)/ \Delta j$$

$$X_0 = (X_{highest} + X_{lowest})/2$$

$$\Delta j = (X - X_0)/r$$

Where:

X_0 = Zero level of both vanadium and phosphorus treatments,

$X_{highest}$ = Highest levels of vanadium and phosphorus treatments,

X_{lowest} = Lower levels of vanadium and phosphorus treatments,

Δj = Change interval of vanadium and phosphorus treatments (change interval for vanadium was 420 and phosphorus change interval was 355),

X = The real/actual concentrations of vanadium and phosphorus treatments and

r = r stand for code values (Table 1, codes column).

2.2. Experiment I

Experiment I was designed with single factor to examine the simple effects of vanadium and phosphorus on plant growth (fresh mass) (Table 1). The treatments levels for this Experiment were as follows: when the action of phosphorus on vanadium was studied, the vanadium concentration was set to zero level as 590 µmol and the phosphorus concentrations were increased from 0 to 1000 µmol by using Sodium dihydrogenorthophosphate (NaH_2PO_4). Similarly, when the effect of vanadium on phosphorus was studied, the phosphorus concentration was set to zero level as 500 µmol and the vanadium concentrations were increased from 0 to 1180 µmol by using ammonium metavanadate (NH_4VO_3). During the experiment, each treatment was replicated five times.

2.3. Experiment II

On the basis of Experiment I, a two factors study (Experiment II) was carried out; each factor was consisted on five levels. Quadratic orthogonal rotation combination design was used to determine the interaction between vanadium and phosphorus in screened genotypes of chickpea. Treatments included in Experiment I were omitted from Experiment II however, both experiments were conducted at the same time with same setup (Table 2). Again, each treatment was replicated five times.

2.4. Plant growth conditions

The seeds of chickpea genotypes C-44 (tolerant to vanadium-stress) and Balkasar (sensitive to vanadium-stress) (Imtiaz et al., 2015b) were purchased from Ayyub Agricultural Research Institute (AARI), Faisalabad, Pakistan. Firstly, the healthy seeds of screened genotypes were sterilized with 0.1% sodium hypochlorite solution for 10 minutes, and washed with distilled water then sown in trays containing sand and irrigated daily with water shower to optimize the water contents. After 10 days, the uniform sized seedlings (5 plants) were transplanted into 4 liters plastic box containing 0.3-strength Hoagland nutrient solution (3.5 liters), twice aeration daily and renew the nutrient solution after 3 days. Hoagland nutrient solution was used in both

experiments and pH of solution was adjusted at 6.5 by adding NaOH/HCl solution. All the chemicals used in these experiments were of analytical reagents. The growth room was climate-controlled with a temperature range 22−25°C and relative humidity 70%. A 14-h photoperiod with an average photon flux density of 820 mmol m^{-2} s^{-1} was supplied by an assembly of cool-white fluorescent lamps.

The seedlings of chickpea genotypes were allowed to grow for 21 days until the effects of applied treatments were fully appeared. The entire plant samples were cut and separated into shoots and roots. Then, shoots and roots were washed with running tap water followed by deionised water. After recording the fresh biomass of shoots and roots, all the samples (shoots and roots) were oven at 100°C for 1 hour and then at 60°C till constant weight to determine the total vanadium and phosphorus.

2.5. Analytical Methods

2.5.1. Phenotypic Characterization and Leaf Anatomy of Chickpea plants

To examine cells viability, newly leaves from both C-44 and Balkasar genotypes were collected and frozen in liquid nitrogen and stored at -80°C until microscopic study. Then, prepare transverse sections of leaves, using free hand sectioning method. Each species samples were investigated for its leaf anatomy by cutting, making temporary slides and mounting transverse sections. For this free hand sectioning and staining techniques was used with plant materials and comfortable with the use of the microscopes. After preparing the slides, two drops of diphenylboric acid 2-aminoethyl ester (DPBA; fluorescent upon binding cells) were put on the slides and examined under Fluorescence microscopy (Zeiss AxioScope.A1, Germany).

2.5.2. Sample preparation and RNA extraction

At harvesting, new leaves were collected from both C-44 and Balkasar genotypes. Samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions, and DNase I (Promega) was used to remove contaminating genomic DNA. The quality of purified RNA was initially evaluated on agarose gel and then quantified using Nano Drop spectrophotometer (Thermo Fisher Scientific, Inc.), and the purity of the total RNA was detected by measuring both the A260/280 and A260/230 ratios. The integrity of RNA samples were further evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). The purified RNA was dissolved in

RNase-free water and stored in a -80°C freezer until subsequent analysis. Purified RNA was used to synthesize cDNA.

2.5.3. Primer design

A relatively short sequence (approximately 150 bp) of Hsp70, Hsp-90, and GAPDH like protein coding transcripts was amplified by using reverse and forward primers. The primers used in the present study were obtained from web-based program (http://www.ncbi.nlm.nih.gov/). The sequences of selected cDNAs are according to genes available on NCBI (National Center for Biotechnology Information) databank. Primers sequences are given in table 3:

2.5.4. Gene expression analysis using semi qRT-PCR

To evaluate the validity of Illumina analysis and assess the expression profiles in terms of specific mRNA abundances, different putative genes were selected randomly and detected by semi qRT-PCR. Reverse transcription reactions were performed using 3 µl total RNA for both genotypes (C-44 and Balkasar) leaf samples. Reverse transcription was performed by super script III Reverse Transcriptase (Invitrogen) and oligo (dT) according to the manufacturer's instructions. Forward and reverse primers were designed by using the Primer 3 software. Beta Actin, as the internal housekeeping gene control, was used to get the bands (25 cycle) using original cDNA. All the cDNA samples were diluted to a concentration which gives same bright bands using the actin primers. Then gene specific primers were used to get different bright bands from different materials (32 cycles). The 10-11 µL reaction mixture contained 0.5 µL (10 mmol) of each primer and 2 µL (50 ng) cDNA was used. Amplification reactions were performed as the following: an initial denaturation step at 94°C for 5 min, 32 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, a final extension at 72°C for 10 min and hold at 25°C. The electrophoresis gel run bands were analyzed to verify the specificity of Semi qRT-PCR.

2.5.5. Vanadium and phosphorus determination

Total vanadium in plant samples was determined by the method used by Hou et al. (2013). The vanadium uptake was determined by using graphite furnace atomic absorption spectrophotometer (GFAAS-GTA 120).

Total phosphorus from plant samples was determined by the method used by Murphy and Riley (1962). After the digestion and dilution, the samples were filtered and read on UV-1600 Spectrophotometer (UED-0806013, Shanghai, China) at 410 nm. The standard curve was drawn

for standards by plotting absorbance against the respective phosphorus concentrations. The phosphorus concentration in plant samples was calculated from standard curve.

3. Statistical analysis

The collected data (fresh biomass) from Experiment I was analyzed with single-factor variance by using Excel 2007 and DPS 7.5 software and significance ($p \leq 0.05$) level among different treatments was analyzed with Tukey method. Data obtained from Experiment II was analyzed to fit following polynomial quadratic equation (Tu and Ma, 2003):

$$y = b_0 + \sum_{j=1}^{m} b_j x_j + \sum_{i \neq j}^{m} b_{ij} x_i x_j + \sum_{j=1}^{m} b_{jj} x_j^2$$

Where:

y = Dependent variable (plant biomass, vanadium uptake or phosphorus uptake),

x = Independent (coded) variable (vanadium or phosphorus),

b_j, b_{ij}, b_{jj} = Regression coefficient,

m = Number of factors and

i, j = Order number of the variables

4. Results and Discussion

4.1. Impact of interaction of vanadium and phosphorus on plant fresh biomass

The fresh biomass of shoots and roots in both chickpea genotypes (C-44 and Balkasar) was declined significantly with the increasing of vanadium levels in nutrient solution when the phosphorus was fixed at 500 µmol. When plants were exposed to fixed phosphorus, 27.65% to 70.64%, 33.54% to 73.44%, 33.06% to 75.13% and 39.79% to 79.25% reduction in fresh biomass of shoots and roots of C-44 and Balkasar was occurred at between 170 to 1180 µmol vanadium, respectively. On the other hand, the shoots and roots fresh biomass was increased when vanadium was fixed at 590 µmol, with increasing phosphorus concentrations in nutrient solution (Table 4). The findings showed that there were almost non-significant results between 170 µmol vanadium and 500 and 1000 µmol of phosphorus and 590 µmol vanadium treatments (Table 4).

Generally, plants use same uptake system for vanadium and phosphorus because both have similar chemical properties (Ding et al., 1994; Rehder, 2003) while, their effects on plants depend upon growth conditions. Previous reports also showed that phosphorus has the ability to mitigate the toxicity of heavy metals and enhance the growth and development of plants (Tu and

Ma, 2003). Our findings are same in line with the results reported by Tu and Ma (2003), Vincenza et al. (2010) and Panuccio et al. (2011). Our results confirmed that when the phosphorus concentration was fixed with increasing vanadium levels then the fresh biomass was decreased while, at higher concentrations of vanadium the fresh biomass was greatly reduced, on the other hand, when vanadium concentration was fixed with increasing phosphorus levels then the trend was completely contrary. The present data showed that at higher levels, phosphorus reduced the uptake of vanadium by both chickpea genotypes while; fixed concentration of phosphorus (500 µmol) reduced the vanadium uptake at lower concentrations of vanadium. The obtained results show that addition of phosphorus in nutrient solution increased the plant fresh biomass as well as phosphorus uptake (Table 4).

4.2. Function model building

Based on the obtained findings, regression model equations between vanadium and phosphorus uptake by chickpea genotypes was established by the use of quadratic regression analysis (Table 5). Each model of regression was tested by analysis of variance, the obtained F and P values are presented in Table 5. Statistical analysis of regression coefficients in vanadium uptake equations Experiment II demonstrated that all the factors were significantly affected vanadium uptake, with linear terms for vanadium and phosphorus uptake in both genotypes. Significant interaction of all factors on plant phosphorus uptake in Experiment II was showed by the linear term for phosphorus ($P \leq 0.005$) and interaction term for vanadium (Table 5).

4.3. Impact of interaction of vanadium and phosphorus on uptake of vanadium and phosphorus

Figures 1 and 2 showed the interaction between vanadium and phosphorus uptake in plants of both chickpea genotypes. The results show that at zero level vanadium and higher level of phosphorus (1000 µmol), both genotypes accumulated plenty of phosphorus however; C-44 was more efficient to accumulate phosphorus than Blakasar (Fig. 1A). The maximum accumulation of phosphorus was observed at higher phosphorus concentration (1000 µmol). At the same time, when the phosphorus was applied at lower concentration with the increasing of vanadium concentrations, the phosphorus accumulation was decreased significantly in both genotypes. Same trend for phosphorus uptake was observed in Balkasar genotype whilst, Balkasar was observed less efficient to uptake phosphorus than C-44 (Fig. 1B). On the other hand, when the chickpea genotypes were exposed to lower phosphorus concentration (0 µmol) with higher

concentration of vanadium (1180 µmol), they accumulated plenty of vanadium and this increase in accumulation of vanadium was linearly increased from lower to higher concentrations (Fig. 2 A & B). With lower vanadium concentration along with increasing phosphorus concentration, the accumulation of vanadium was decreased effectively. Overall, results show that higher concentrations of vanadium reduced the uptake of phosphorus while, higher concentrations of phosphorus inhibited the uptake of vanadium by both chickpea genotypes.

Statistically, significant decline in vanadium uptake with increasing concentrations of phosphorus was observed. The observed reduction in vanadium uptake by both genotypes of chickpea might be due to efficient and readily uptake of phosphorus than vanadium by phospahte-carriers located in root plasma membrane or due to formation of insoluble complex of vanadium and phosphorus in the root circumference environment (Marschner, 1995) because vanadate and phosphate have same chemical structure therefore, both ions taken up via same carriers by plants. Therefore, vanadium and phosphorus exert antagonistic effects on each other during uptake and transport (Ding et al., 1994). The obtained results of the present study also confirmed that C-44 accumulated higher vanadium than Balkasar; these findings are same in line with the Imtiaz et al. (2015b). In fact, inhibition of phosphorus uptake due to higher concentrations of metals is common phenomenon in phytoplankton (Planas and Healey, 1978), and terrestrial angiosperms such as barley (*Hordeum vulgare* L.) (Asher and Reay, 1979) and velvet grass (*Holcus lanatus* L.) (Meharg et al., 1994). And at higher levels, vanadium affects the growth, photosynthesis, induces DNA damage and also reactive oxygen species (ROS), and integrates cell membrane and roots which can be major reasons for reduction in phosphorus uptake (Macara et al., 1980; Imtiaz et al., 2015b; Imtiaz et al., 2016). On the other hand, inhibition of metals by phosphorus was also reported in wheat (Scheffer and Schachtschabel., 2002), in cabbage (Wang et al., 2008), in cauliflower (Chen et al., 2009) and in chickpea (Aydin et al., 2009). There are various mechanisms to immobilize the heavy metals, apart from the mechanisms where phosphorus help to precipitate metals ions (Aikpokpodion et al., 2012). Moreover, our results were confirmed from the analysis of genes expression; at higher concentration of vanadium with fixed phosphorus concentration stressed gene (Hsp70) was upregulated while, at higher concentration of phosphorus with fixed vanadium concentration the genes were not expressed, it might be due to inhibition of vanadium uptake by phosphorus.

4.4. Impact of interaction of vanadium and phosphorus on morphological changes in leaves of chickpea plants

The morphology of leaves of both genotypes was assessed following hydroponic culture with vanadium and phosphorus under fluorescence microscopy. Generally, cell death can occur by necrosis or programmed cell death (PCD) (Clarke et al., 2000) and heavy metals stress can induce PCD and necrosis in plants (Miloslava and Ales, 2000; Imtiaz et al., 2016). To assess the cell death induced by vanadium and either phosphorus has ability to remediate the vanadium stress via cell death, the leaves samples were examined by diphenylboric acid 2-aminoethyl ester (DPBA; fluorescent upon binding cells) staining under Fluorescence microscopy (Fig. 3). The effects of vanadium and phosphorus exposure on leaves morphology were varied by genotypes. The leaves samples treated with zero vanadium showed healthy and clear with good forms of cells while, the leaves treated with higher vanadium (1180 µmol) and lower phosphorus (500 µmol) showed deformed, disturbed and reduced sized cells in leaves of both chickpea genotypes. However, the proportion of deformation of cells was higher in Balkasar as compared to C-44 genotype. Moreover, when leaves were treated with elevated phosphorus (1000 µmol) and lower vanadium (590 µmol) showed better results than elevated vanadium concentration and the proportion of cells deformation was lower as compared to higher vanadium concentration and lower phosphorus (Fig. 3). While, exposure of vanadium induces alterations in plant metabolism and can causes cell death. Previous studies also reported that Cd induces cell death in plants within a few hours after exposure (Piqueras et al., 1999; Olmos et al., 2003; Garnier et al., 2006). Cadmium induced necrotic type of cell death in tobacco cell culture and onion root apical cells; the cell death was slower at lower doses of Cd and illustrates characteristics typical of PCD (Miloslava and Ales, 2000; Behboodi and Samadi, 2004; He et al., 2011). Moreover, Liu et al. (2015) also reported that application of phosphorus alleviated the toxic effects induced by abiotic stress in plants. In our experiment, C-44 was exhibited more tolerance than Balakasar to vanadium stress. At higher doses, however, a process of PCD was triggered, as indicated by deformation and reduction in size of cells. On the other hand, higher phosphorus and lower vanadium showed little cell death than higher vanadium dose. The obtained results show that vanadium-induced cell death was same with the findings of Roberto et al. (2009) and Imtiaz et al. (2016).

4.5. Impact of interaction of vanadium and phosphorus on heat shock genes expression

Among all the abiotic stresses, heavy metals stress is one of the major reasons to up regulate the genes especially “heat shock genes” (HSGs) which encode heat shock proteins (HSPs) and their expression is much necessary for the survival of plants under metals toxicity (Chang et al., 2007). Heat shock proteins induce significant variations in their expression under a variety of stressed conditions including metals (Khalid et al., 2014). The HSPs family can be use as metals toxicity biomarker (Cochrane et al., 1991). The genes related to HSPs are extremely heterogeneous in nature and dynamic family of protein (Prasinos et al., 2005). Among the plants, HSPs are grouped into 5 different families: HSP100, HSP90, HSP70, HSP60 and HSP20 (Swindell et al., 2007), while only HSP70, HSP60 and HSP90 proteins are considered as more important and conserved proteins in nature and play fundamental role in response to abiotic stress (Kulz, 2003). The glyceraldehydes 3-phosphate dehydrogenase (GAPDH) plays important role in living organism (Figge et al., 1999) and found abundantly in cells and served as model protein for researchers studying functions and metabolic pathways (Sirover, 1999). The GAPDH involved in DNA replication and repair and phosphotransferase activity (Tatton et al., 2000). Previous studies report that GAPDH also involved in pathways related to energy production and physiological male sterility of wheat plants and inhibition in expression of GAPDH can cause energy shortage during pollen development and ultimately lead to pollen abortion (Wei et al., 2009). Moreover, GAPDH can also be used as biomarker against abiotic stress in plants (Qi et al., 2011). GAPDH also used as biomarker to stress environment because it is localized in nuclear part of cells which undergo apoptosis (Saunders et al., 1999; Zubin and Jean-Luc, 2001).

A range of concentrations of two elements vanadium and phosphorus was used in the present study to evaluate the responses of HSPs in chickpea genotypes. To determine the expression levels of HSP70, HSP90 and GAPDH genes in leaves of chickpea genotypes: C−44 (tolerant to vanadium-stress) and Balkasar (sensitive to vanadium-stress), real-time PCR was performed (Fig. 4). The leaves samples were only selected from control treatment, and treated with higher vanadium (1180 µmol) with fixed phosphorus (500 µmol) and higher phosphorus (1000 µmol) with fixed vanadium (590 µmol) concentrations of both genotypes. Transcript levels were normalized with housekeeping Actin transcripts (Fig. 4). The obtained bands of gel electrophoresis confirmed that HSP90 and GAPDH showed similar expression patterns in both genotypes however, HSP90 showed little more expression at phosphorus @ 1000 µmol + vanadium @ 590 µmol and vanadium @ 1180 µmol + phosphorus @ 500 µmol treatments in

C−44 plants which indicate that they may be functionally redundant (Fig. 4). When C−44 plants were treated with phosphorus @ 1000 µmol + vanadium @ 590 µmol treatment, the HSP90 gene expression was lower than vanadium @ 1180 µmol + phosphorus @ 500 µmol treatment. At the same time, there was non-significant difference for GAPDH expression patterns in both chickpea genotypes. The results about HSP70 expression patterns were different than HSP90 and GAPDH genes. The only treatment vanadium @ 1180 µmol + phosphorus @ 500 µmol showed HSP70 band in C−44 plants, all other treatments showed no expression at any concentration in both genotypes (Fig. 4). The HSP70 expression patterns showed that at first, the stress response looks limited but at higher vanadium, HSP70 protein showed response against vanadium toxicity only in C−44 plants.

Heat shock proteins (HSPs) are important proteins which play fundamental roles in protein metabolism, and translocation, in stressed environment and developmental response (Xu et al., 2010). HSPs are used as biomarkers because these are induced in stressed conditions (Goupil et al., 2009), therefore these are much more sensitive to stress than traditional parameters. The bands generated of expression patterns of HSP90 are same in line with the results reported in rice seedlings when exposed to various abiotic stresses by Pareek et al. (1995) and Liu et al. (2006). The similar stress related findings in plants were also obtained by Milioni and Hatzopoulos (1997) and Goupil et al. (2009) under heavy metals stress. The expression patterns of HSP70 in the present study also show an agreement with the results of Sugino et al. (1999), Alvim et al. (2001), Rizhsky et al. (2002), Tomanek and Sanford (2003), Oono et al. (2003), Cho and Hong 2004, Kim et al. (2005) and Alakananda et al. (2010). Eun and Choo (2006) also reported that HSP70 protein was over expressed when tobacco plants were exposed to abiotic stress. He et al. (2015) also reported that plants respond differently to Cd stress because of genes expression involved in detoxification and mediation of various enzymes to alleviate the metals stress. These results could demonstrate that higher concentrations of vanadium might be involved in destabilization of cellular mechanism and damage it. Moreover, our results also suggest that over expression of HSPs can confer vanadium stress tolerance. However, further research is required to find the pathway through which HSPs were over expressed under vanadium stress.

In summary, it can be concluded that plants fresh biomass was significantly influenced by vanadium and phosphorus application. The results also indicated that higher concentrations of vanadium were toxic while; addition of phosphorus was beneficial to chickpea plants. Our

findings also confirmed that lower doses of vanadium promoted the phosphorus uptake in chickpea plants whilst; higher levels of vanadium inhibited the uptake of phosphorus in both genotypes of chickpea. Higher concentrations of phosphorus in growth medium significantly reduced the vanadium uptake by chickpea genotypes. The results about morphological changes in leaves indicated that the cells were deformed and reduced in size when treated with higher concentration of vanadium with fixed phosphorus however, there was little deformation and reduction in cells size were observed when plants were treated with higher concentration of phosphorus with fixed vanadium. In general, the proportion of deformation of cells was higher in Balkasar as compared to C-44 genotype. The generated results also showed that at elevated vanadium with fixed phosphorus, Hsp70 was expressed only in C-44 genotype. Moreover, further studies are needed to know the mechanistic explanation of genes expression and inter-relationship of vanadium and phosphorus in plants that are currently being unraveled.

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Competing interests

None of the authors have any competing of interest.

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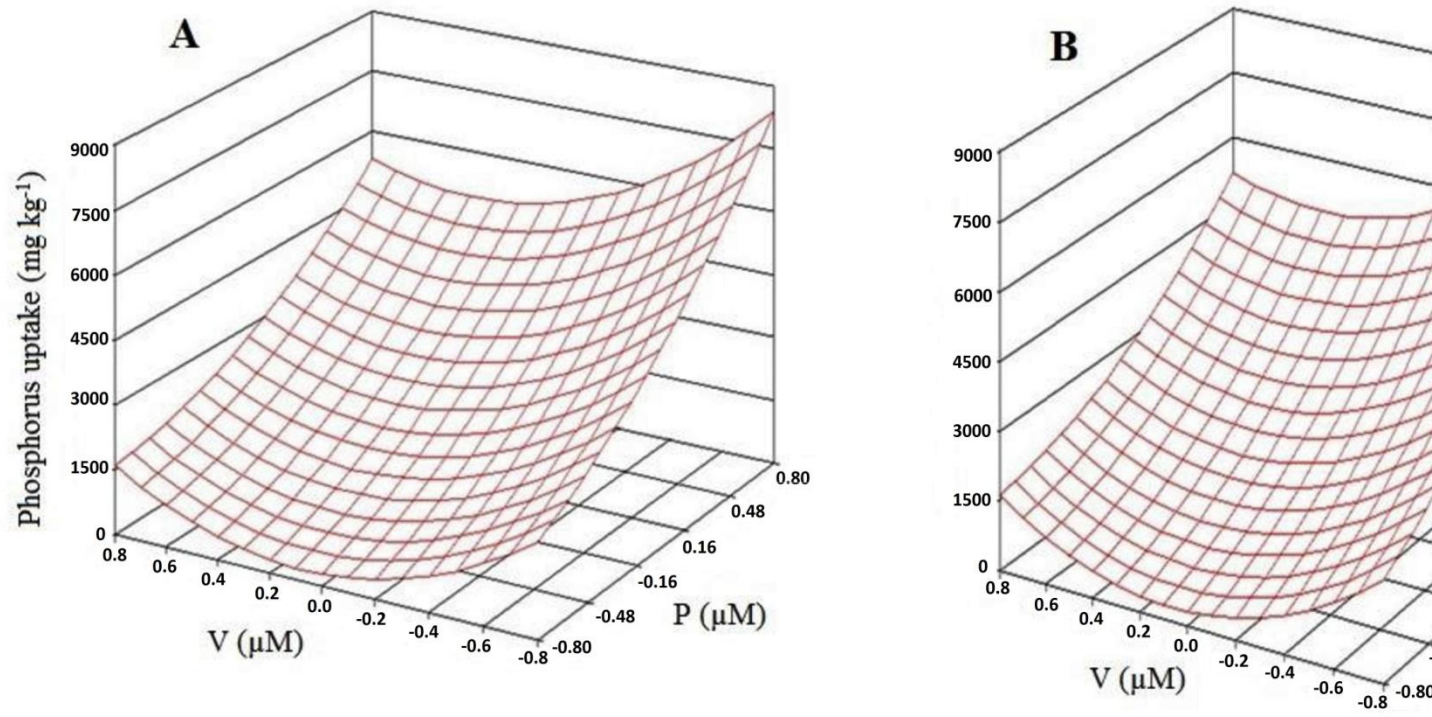

Fig. 1 Three-dimensional plot describing the interactive effects of vanadium (V) and phosphorus (P) on phosphorus uptake in (A) C-44 and (B) Balkasar genotypes of chickpea (five plants in each box) were grown hydroponically for 21 days in Experiment II. The plot was drawn from second-order equation of phosphorus uptake by imposing the central value of 500 µmol for phosphorus. ANOVA was significant at $P<0.0002$.

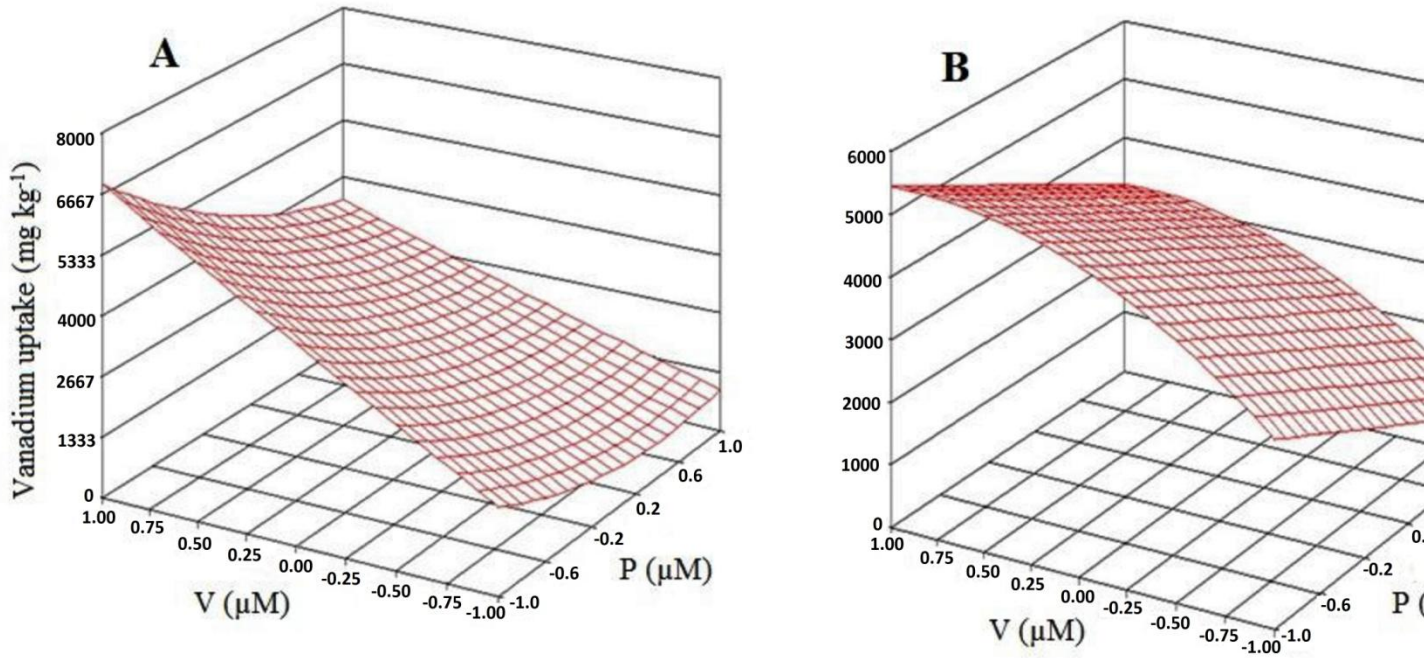

Fig. 2 Three-dimensional plot describing the interactive effects of vanadium (V) and phosphorus (P) on vanadium uptake in (A) C-44 and (B) Balkasar genotypes of chickpea (five plants in each box) were grown hydroponically for 21 days in Experiment II. The plot was drawn from second-order equation of vanadium uptake by imposing the central value of 590 µmol for vanadium. ANOVA was significant at $P<0.0002$.

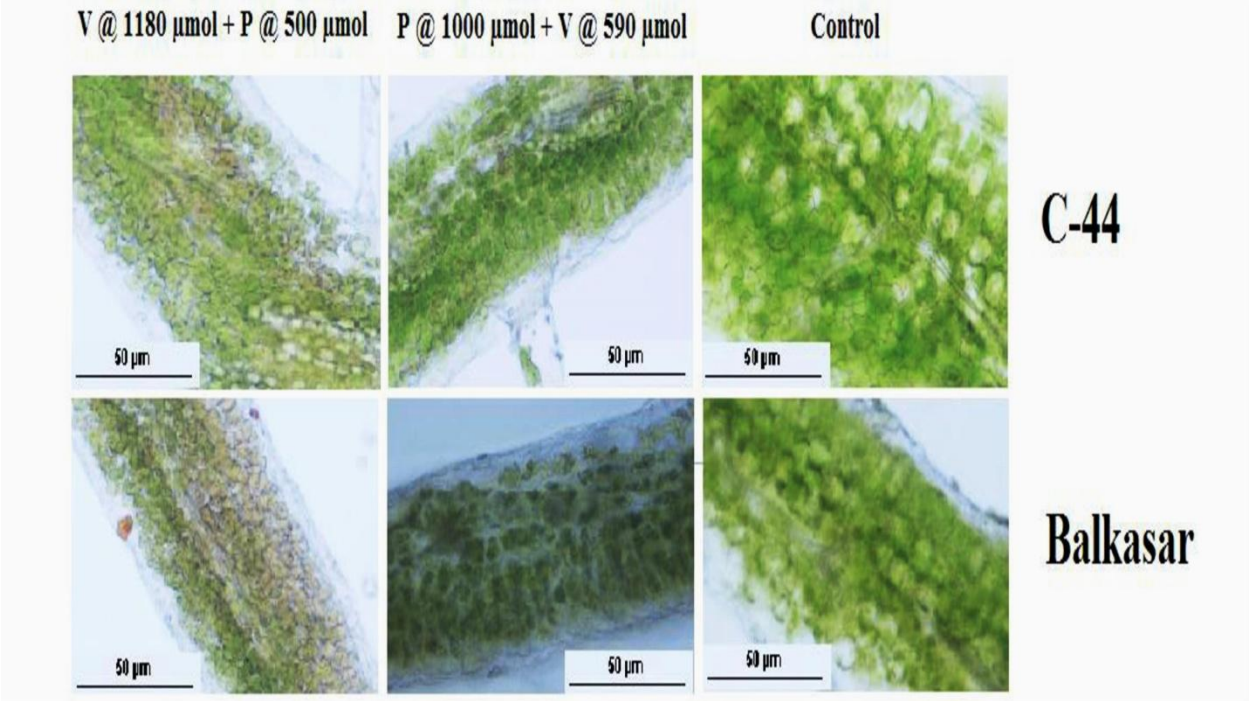

Fig. 3 Transverse sections study of leaves of chickpea genotypes: C−44 and Balkasar under fluorescence microscopy. The combined effects of vanadium and phosphorus on the leaves morphology; healthy and large sized cells: control treatment, little damage, deformed and reduced in size cells: phosphorus @ 1000 µmol + vanadium @ 590 µmol treatment and severe damage, deformed and reduced in size cells: vanadium @ 1180 µmol + phosphorus @ 500 µmol treatment. The leaves samples were only selected from the plants exposed to control treatment, higher phosphorus with fixed vanadium and higher vanadium with fixed phosphorus concentrations.

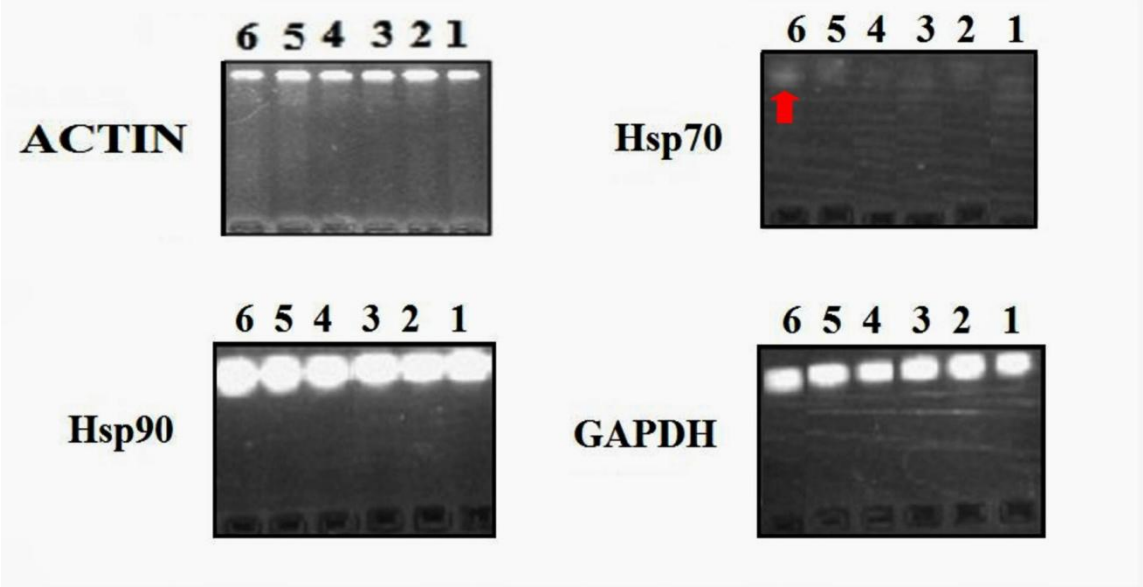

Fig. 4 Expression patterns of HSP70, HSP90 and GAPDH genes in chickpea genotypes: C-44 and Balkasar grown in hydroponic conditions under vanadium and phosphorus for 21 days. Lane 1, RNA from untreated Balkasar seedlings (control); Lane 2, RNA from phosphorus @ 1000 µmol + vanadium @ 590 µmol treated Balkasar seedlings; Lane 3, RNA from vanadium @ 1180 µmol + phosphorus @ 500 µmol treated Balkasar seedlings; Lane 4, RNA from RNA from untreated C-44 seedlings (control); Lane 5, RNA from phosphorus @ 1000 µmol + vanadium @ 590 µmol treated C-44 seedlings; Lane 6, RNA from vanadium @ 1180 µmol + phosphorus @ 500 µmol treated C-44 seedlings.

Table 1 Treatment concentrations of vanadium and phosphorus for Experiment I to study the simple effects on chickpea genotypes grown hydroponically

Treatment levels	Codes	VO_4(µmol)	PO_4 (µmol)
1	–1.414	0	0
2	–1	170	146
3	0	590	500
4	+1	1010	855
5	+1.414	1180	1000

The treatment concentrations were computed according to the codes of two factors, five levels central composite design. Each treatment was replicated five times and the experiment was conducted by imposing the central values of two factors, which were 590 µmol for vanadium and 500 µmol for phosphorus.

Table 2 Treatments levels of vanadium and phosphorus for Experiment II, to study their interactive effect on chickpea genotypes grown hydroponically

Run	Codes		Treatments (µmol)	
	Vanadium	Phosphorus	Vanadium	Phosphorus
1	+1	+1	1010	855
2	+1	–1	1010	146
3	–1	+1	170	855
4	–1	–1	170	146
5	+1.414	0	1180	500
6	–1.414	0	0	500
7	0	+1.414	590	1000
8	0	–1.414	590	0
9	0	0	590	500
10	0	0	590	500
11	0	0	590	500
12	0	0	590	500
13	0	0	590	500
14	0	0	590	500
15	0	0	590	500
16	0	0	590	500
17	–1	0	170	500
18	+1	0	1010	500
19	0	–1	590	146
20	0	+1	590	855

The treatments were calculated on base of two factors and five levels central composite designed.

Table 3 Primers sequences of selected genes

Primers	Primers sequence
HSP70	Fw: 5′−ACGTGGTCGCGGCCGAGGT
HSP70	Rev: 5′−ACGTGGTCGCGGCCGAGGT
HSP90	Fw: 5′−GCAGCATGGCTGGTTACATGT
HSP90	Rev: 5′−TGATGGGATTCTCAGGGTTGA
GAPDH	Fw: 5′−CCAAGGTCAAGATCGGAATCA
GAPDH	Rev: 5′−CAAAGCCACTCTAGCAACCAAA

Table 4 Fresh biomass yield of chickpea genotypes seedlings exposed to various concentrations of vanadium and phosphorus when phosphorus was fixed with increasing vanadium and vanadium was also fixed with increasing phosphorus in hydroponic solution. Values are mean ± SD (n=5). Means contained similar letters in column are not significantly (*P≤0.05*) different according to Tukey method.

Treatments (µM)	C–44		Balkasar	
	Shoots, *FW(g)	Roots, FW(g)	Shoots, FW(g)	Roots, FW(g)
Control	0.991a	0.960a	0.965a	0.935a
170 (V) + 500 (P)	0.717bc	0.638bc	0.646b	0.563b
590 (V) + 500 (P)	0.494ef	0.450ef	0.455de	0.391de
1010 (V) + 500 (P)	0.400g	0.312g	0.328g	0.282g
1180 (V) + 500 (P)	0.291i	0.255hi	0.240hi	0.194hi
146 (P) + 590 (V)	0.362h	0.280gh	0.266h	0.244h
500 (P) + 590 (V)	0.505e	0.461e	0.455d	0.390d
855 (P) + 590 (V)	0.582d	0.569d	0.374f	0.307f
1000 (P) + 590 (V)	0.734b	0.664b	0.544c	0.490c

Note: *The weight represents the mean of 5 replications for each genotype, *FW = fresh weight*

Table 5 Best-fit equations and statistical tests relative to the effects of independent variables vanadium and phosphorus and the uptake of vanadium and phosphorus by chickpea genotypes: C-44 and Balkasar

Equations of actual variables
Vanadium uptake (C-44 plants)
$y_1 = 2719.72+1878.96[V]-1095.36[P]+36.75[P][P]+529.06[V][V]-617.92[V][P]$, $F = 26.72$ $p< 0.0001$
Phosphorus uptake (C-44 plants)
$y_2 = 2039.84-1141.17[V]+3106.66[P]+2503.92[P][P]+1117.33[V][V] -982.17[V][P]$, $F = 16.36$ $p< 0.0002$
Vanadium uptake (Balkasar plants)
$y_3 = 3750.54+1249.78[V]-1102.42[P]-685.43[P][P]+1.78[V][V]-14.67[V][P]$,

F = 14.78 p< 0.0002

Phosphorus uptake (Balkasar plants)

y_4 = 2035.45-1145.40[V]+3052.65[P]+2461.69[P][P]+1099.53[V][V]−1033.42[V][P],

F = 18.27 p< 0.0001