Efficacy of a multi-dentate Schiff base and its vanadyl complex on various morphological and biochemical parameters of *Vigna radiata* L.

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**ABSTRACT**

A \(\text{N}_2\text{O}_2\) donor Schiff base polydentate ligand was synthesized by the condensation of benzidine and benzil in ethanolic medium. The formed ligand was condensed further with \(\text{VOSO}_4\cdot\text{H}_2\text{O}\) to get the corresponding vanadyl complex. Both the ligand and the complex were characterized by spectroscopic and elemental analyses. Mung bean (*Vigna radiata* L.) was selected as a plant material. Various morphological and biochemical parameters, e.g. leaf senescence assay, chlorophyll content, and different reactive oxygen species (ROS) were estimated and were compared to those with ammonium vanadate. Outcomes of the experiments revealed that the Schiff base complex has less toxic effects than ammonium vanadate on mung bean seedlings and provide better tolerance to vanadium toxicity. Though different stress marker and ROS accumulation were less and minimum pigment damage was noticed in the Schiff base complex-treated seedlings but the optimum positive impact largely depends on the dose. Beyond certain concentration, the complex may show inhibitory effects on the plants. Therefore, the present study revealed that heavy metal Schiff base complexes can be used as potential supplement to meet up micronutrient deficiency and at the same time such complexes can minimize the toxicity generated by application of different heavy metals.

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**Introduction**

Vanadium, one of the important trace elements for plants is found scattered in the environment through the leaching of rocks, combustion of coal or petroleum products, and residual slag from the steel industry (Baken et al. 2012). Although Vanadium (V) was discovered in early eighteenth century, very little work was done on V before the 1950s (Bertrand 1950). Initial studies showed that V is toxic for most of the plant species and that is why there was very little interest in evaluating its effect on plants (Witz and Osmond 1886). Interest intensified when Arnon and Wessel (1953) concluded that V is essential for some plants (Trejo-Téllez Gómez-Merino, and Álcantar-González 2016). Subsequent studies showed that V is generally toxic to terrestrial plants when applied in amounts greater than pico-molar levels (Davis, Beckett, and Wollan 1978). However, it is found to be beneficial for plant growth and development when applied in trace amount (Prarr 1966; Welch 1973). Further studies indicated that V due to its various oxidation states (\(-1\) to \(+5\)), the toxicity varies. It was also found that the pentavalent state (V\(^{+5}\)) is more toxic than...
the corresponding tetravalent state (Tian, Yang, and Huang 2015). Moreover V$^{4+}$, predominantly found in the soil, is responsible for the development of plants (Larsson et al. 2013). Study revealed that V acts as constituent of the cofactors in vanadate-dependent haloperoxidases and vanadium nitroginate (Hu, Lee, and Ribbe 2012). Many important electron transfer processes and plasma membrane hydrogen (H$^+$)-translocation ATPase are largely dependent upon V (Vara and Serrano 1982). The monomeric form of vanadate is both structurally and electronically identical with phosphate. This facilitates vanadate to inhibit or to activate the enzymes that interact with phosphorylated species (Akabayov and Akabayov 2014). Generally, vanadium-containing fertilizers (e.g. NH$_4$VO$_3$) are used to provide V to plants in optimum level. These types of fertilizers, being ionic in nature, are responsible for the alteration of pH of soil (Thind and Rowell 1999). So nowadays, more emphasis is given to metal chelates that are less reactive and can solve the V deficiency as well as V toxicity for longer period of time without hampering the medium (Khoshgoftarmanesh et al. 2010; Wallace and Wallace 1982). Inspired by these facts, a polydentate (N$_2$O$_2$ donor type) Schiff base ligand and its oxo vanadium complex have been synthesized and their effects on Vigna radiata L. were thoroughly monitored. Mung bean (V. radiata L.) is chosen as plant material because of its global importance as pulse. It is grown in South, East, and Southeast Asia where 90% of global production currently occurs (Tomooka et al. 1992). Mung bean provides significant amounts of protein, carbohydrates, and a range of micronutrients in diets. Its cultivation is also important as it maintains the soil fertility through nitrogen fixation. In this study, the responses of vigna seedlings to vanadium toxicity when exposed to ammonium vanadate and vanadium Schiff base complex are thoroughly monitored in terms of relative water content, biochemical components, oxidative stress markers, and overall tolerance level in vigna plants.

Materials and methods

**Synthesis of tetradentate N$_2$O$_2$ donor Schiff base ligand and its vanadium(IV) complex**

Ethanolic solution of benzidine (purity > 99%, procured from Sigma-Aldrich, Germany) was refluxed with benzil (purity l > 98%, procured from Sigma-Aldrich) in a round-bottom flask in 1:2 molar ratio for ~4 hr at temperature of 40–60°C with constant stirring to get the olive green colored ligand L$_1$ [C$_{40}$H$_{28}$N$_2$O$_2$. 2H$_2$O]. The ligand was filtered off, washed, and recrystallized from ethanol. Then it was dried in a vacuum desiccator. To synthesize vanadium(IV) complex, the ligand was further refluxed with an ethanolic solution of VOSO$_4$.xH$_2$O (procured from Sigma-Aldrich) for ~8 hr at 40–60°C in a molar ratio of 1:1 with constant stirring. The complex C$_1$ obtained with a molecular formula of C$_{96}$H$_{56}$N$_4$O$_6$V$_2$.5H$_2$O was washed with ethanol and dried over anhydrous CaCl$_2$. Both the synthesized ligand and the complex were characterized by elemental microanalysis, infrared, and electronic spectroscopic spectra. These results were similar to those reported earlier in the literature (Ahmed and BenGujji 2009).

**Maintenance of plants**

Vigna seeds were surface sterilized using 1% (wt/vol) sodium hypochlorite solution and rinsed with double distilled water. Seeds were then transferred to plastic pots (diameter 11 cm) containing sterile soil. Each pot contained five seeds and the pots were kept in temperature of 25 ± 2°C for a photoperiod of 8 hr with 65%–70% relative humidity regime. Seedlings were watered regularly every alternate day and after one month plants were utilized for further experiments. After a growth period of 30 days, the plants in their vegetative phase were taken, roots were gently washed with sterile H$_2$O and transferred to 20% Steiner nutrient solution (1.8 mM Ca(NO$_3$)$_2$.4H$_2$O, 0.8 mM MgSO$_4$.7H$_2$O, 0.2 mM KH$_2$PO$_4$, 0.6 mM KNO$_3$, 0.6 mM K$_2$SO$_4$, 89.31 μM Fe,
42.37 μM Mn, 7.12 μM Zn, 39.98 μM B, 2.93 μM Cu, 1.80 μM Mo). The plants were then allowed to acclimatize in this solution for 48 h. After 48 h of acclimation, this nutrient solution was entirely replaced and treatments were applied in the renewed nutrient solution with different concentrations (5, 10, and 20 μM V) of Schiff base ligand (L1), Schiff base vanadyl complex (C1), and NH4VO3 (AV) along with a control (no treatment of ligand, Schiff base complex, NH4VO3 in nutrient solution) separately for 7 days. Each treatment had three replicate sets and experiment was conducted in randomized design method. After 7 days, leaf samples were collected, frozen in liquid nitrogen, and subsequently used for biochemical tests. The fresh weight of seedlings was taken immediately after sampling to avoid any water loss from leaf samples.

**Growth parameters**

Plants from different treatment sets were harvested and weighed to get fresh biomass (F.W.). The samples were then absorbed to full turgidity for 6 hr at 25°C to get turgid weight (T.W.). Subsequently, the samples were parched in a hot air oven at 70°C for 48 h to get dry biomass (D.W.) of each sample. Relative water content (RWC) was calculated by using the method of Farooqui et al. (2000).

**Cell viability**

Briefly, 10-mm leaf disc from the control and the treated plants were kept in glass vials with 1% MTT (i.e. 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution in dark for 12 hr. Leaf samples were placed in 5% alcohol and kept for boiling till all the alcohol evaporated off. Thereafter, the absorbance of the purple colored extract was measured at 485 nm.

**Electrolyte leakage and membrane injury**

Electrolyte leakage was measured as per Yan et al. (1996). Leaves from each treatment were washed carefully with deionized water, kept in culture tubes containing 10 mL of deionized water and incubated at 25°C on a rotary shaker for 24 h. The electrical conductivity of the solution (C1) was measured with a conductivity meter. Then the samples were autoclaved at 120°C for 20 min and cooled to room temperature prior to assessing the final electrical conductivity (C2). Electrolyte leakage was measured as follows:

$$EC(\%) = \frac{C_1}{C_2} \times 100$$

Membrane lipid peroxidation was assessed in terms of malondialdehyde (MDA) content according to Heath and Packer (1968). Fresh leaves (0.5 g) were ground in precooled 0.1% (wt/vol) trichloroacetic acid (TCA) followed by centrifugation at 10,000 rpm for 15 min at 4°C. About 0.5 mL of the slurry was mixed with 2 mL of 0.5% Thiobarbituric acid (TBA) in 20% TCA, followed by heating for 30 min at 95°C and subsequent cooling on ice. The absorbance of the reaction mixture was measured at 532 and 600 nm and the MDA content was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

**Determination of free amino acids and total sugar**

From the ethanol extraction of leaves, 500 μL were mixed with 500 μL of ascorbic acid and sodium citrate buffer mix (0.2% wt/vol), at a pH of 5.2 and 1000 μL of ninhydrin (1% wt/vol) in ethanol at 70% (vol/vol) was added. The samples were kept in a water bath at 95°C for 30 min
and followed by cooling at room temperature. The free amino acids were measured in a spectro-
photometer using leucine (10 mM in ethanol 70%) as standard at 570 nm.

To measure total soluble sugar, leaves (0.5) were homogenized in 10 mL of 95% ethanol
(Harborne 1973). Then 1 mL of extract and 4 mL of anthrone reagent (0.2%) were kept in boiling
water bath for 10 min followed by quick cooling. Then absorbance was taken at 620 nm and
quantified using glucose as standard (Plummer 1978).

H$_2$O$_2$ content

H$_2$O$_2$ levels in the leaves were estimated according to Jana and Choudhuri (1982) with minor
modifications. H$_2$O$_2$ levels were calculated using extinction coefficient 0.28 $\mu$mol$^{-1}$ cm$^{-1}$.

Chlorophyll content

Chlorophyll was extracted from the leaves using 80% (vol/vol) acetone according to Lichtenthaler
(1987). Absorbance was taken spectrophotometrically using at 480 nm, 645 nm, and 663 nm. Total
chlorophyll, chlorophyll a, and chlorophyll b were calculated using following formula (Arnon
1949):

\[
\text{Total chlorophyll} = (20.2A_{645} + 8.02A_{663}) \text{mgg}^{-1}\text{F.W.}
\]

\[
\text{Chlorophyll a} = (12.7A_{663} - 2.69A_{645}) \text{mgg}^{-1}\text{F.W.}
\]

\[
\text{Chlorophyll b} = (22.9A_{645} - 4.68A_{663}) \text{mgg}^{-1}\text{F.W.}
\]

Leaf disc bioassay

The fully expanded and fresh leaves from the plants were gently washed in deionized water and
1-cm diameter leaf discs were then floated in a 5 mL solution of schiff base ligand (L1), Schiff
base VO(II) complex (C1), and NH$_4$VO$_3$ (AV) for 6 days. Leaf discs floated in sterile distilled
H$_2$O served as the experimental control The effects of different complexes on leaf discs were
assessed on the basis of the phenotypic alteration especially leaf color (Fan, Zheng, and
Wang 1997).

Statistical analysis

Data were analyzed by using standard error and Least Significant Difference (LSD) tests at
$p \leq 0.05$ probability level using IBM SPSS statistics 21 software.

Results and discussion

Characterization of the prepared ligand and its vanadyl complex

The analytical and spectral data recorded for the Schiff base (L1) and its vanadyl complex (C1)
was found to be almost as same as reported in the literature (Ahmed and BenGujji 2009). Some
Table 2. Some characteristic infrared (in cm$^{-1}$) and electronic spectral (in nm) data of the ligand (L1) and its vanadyl complex (C1).

|       | $\nu_{C=N}$ | $\nu_{O-H}$ | $\nu_{C=O}$ | $\nu_{V-O}$ | $\nu_{V-N}$ | $\lambda_{\text{max}}$ |
|-------|--------------|--------------|--------------|--------------|--------------|------------------------|
| L1    | 1620         | 3375         | 1731         | $\sim$       | $\sim$       | 263.1                  |
| C1    | 1624         | 3223         | 1731         | 982          | 491          | 844.7, 445.1, 367.8    |

of the characteristic analytical and spectral data and scheme of reaction are listed in Tables 1 and 2 and in Supplementary Material. Characteristic Infrared (IR) bands at 1620, 1731, and 3375 cm$^{-1}$ appeared due to $\nu_{C=N}$, $\nu_{C=O}$, and $\nu_{O-H}$ vibrations, respectively for the ligand (L1). After complexation the $\nu_{C=N}$ band shifted to 1624 cm$^{-1}$ due to coordination bond formation. For the complex, two new bands appeared at 982 and 491 cm$^{-1}$ due to $\nu_{V-O}$ and $\nu_{V-N}$ vibrations, respectively. The electronic spectra were measured with $5 \times 10^{-4}$ molar solutions in dimethylformamide for both the compounds. The ligand (L1) has a characteristic $\lambda_{\text{max}}$ at 263.1 nm due to $\pi-\pi^*$ transition and its vanadyl complex (C1) manifested three peaks at 844.7, 445.1, and 367.8 nm, respectively due to characteristic transitions as reported earlier in the literature (Ahmed and BenGujji 2009).

**Effects of the ligand and its vanadyl complex on vigna seedlings**

**Leaf disc bioassay**

V sensitivity of mung leaf was determined by leaf disc senescence bioassay. It is represented in terms of degree of leaf decoloration and percentage (%) decrease of the chlorophyll content of the detached leaves at the concentration range of 5$\mu$M, 10$\mu$M, and 20$\mu$M of L1, C1, and NH$_4$VO$_3$ in comparison to the detached leaves kept in sterile distilled water. For control and L1-treated Vigna seedlings, the leaf color and chlorophyll content were almost alike after 7 days of treatment indicating that L1 ligand do not have negative impact on seedlings (Figures 1 and 2). The leaf discs turned slightly blackish when kept at 20$\mu$M concentration of NH$_4$VO$_3$ for 7 days. On the contrary, leaf decoloration was found to be least for C1-treated leaf discs indicating less negative impact of the Schiff base complex (C1) on seedlings chlorophyll.

**Fresh biomass, dry biomass and relative water content**

Plants from different treatment sets were harvested and weighed to get fresh biomass. Subsequently, to get dry biomass the samples were parched in a hot air oven at 70$^\circ$C for 48 h. The outcomes reveal that plants treated with the complex (C1) were able to retain higher percentage of fresh mass and dry mass over the period of time than NH$_4$VO$_3$-treated plants with increasing concentration suggesting less toxicity of the Schiff base complex (C1) (Figures 3 and 4). Same trend has been observed for relative water content (RWC). Drastic decrease in relative water content at higher concentration for NH$_4$VO$_3$-treated plants signified higher stress in cells (Table 3).

**Oxidative stress**

Plants facing adverse conditions produce reactive oxygen species (ROS) at vital cell organelles like chloroplast, mitochondria, and peroxisomes. These ROS are formed as a byproduct of plant aerobic metabolism (Huang et al. 2016; Dietz, Turkan, and Krieger-Liszkay 2016; Sandalio and Romero-Puertas 2015). Amongst a variety of ROS, H$_2$O$_2$ is highly stable and can remain in cell causing damage to cell viability and induce senescence. ROS also increases lipid peroxidation in both cellular and organelle membranes and thus further induce membrane injury, protein degradation, and thereby affects photosynthesis (Sarkar, Chakraborty, and Chakraborty 2018; Huang et al. 2019). Lipid peroxidation produces malonaldehyde as the end product in a chain of reactions with membrane phospholipid molecules (Huang et al. 2019). Application of beneficial
elements in a dose-dependent manner can modulate metabolic functions positively to favor plant vigor development. These effects can further vary depending on dose frequency, chemical form, and genotypes. Previous reports have suggested many basic elements including vanadium salt (mainly NH₄VO₃) when applied in low concentrations can positively stimulate various plant functions and increase plant vigor and biomass but these elements may induce cellular toxicity and stress at high concentration (Pilon-Smits et al. 2009; Tang et al. 2009; García-Jimeñez...
et al. 2018). To have a better understanding of the adverse impact of different concentrations of the Schiff base VO$_2^{+}$ complex (C1) and NH$_4$VO$_3$ (AV) on plants, stress markers such as electrolyte leakage (EL), hydrogen peroxide (H$_2$O$_2$), and peroxidation of membrane lipids (MDA) and cell viability were assessed (Figure 5).

Figure 3. Effects of the control, L1, C1, and NH$_4$VO$_3$ (AV) on fresh biomass of mung bean at various concentrations. Values are represented as mean ± SD (n = 3). Bars with different letters are significantly different at $p \leq 0.05$ according to Fishers LSD set.

Figure 4. Effects of the control, L1, C1, and NH$_4$VO$_3$ (AV) on dry biomass of mung bean at various concentrations. Values are represented as mean ± SD (n = 3). Bars with different letters are significantly different at $p \leq 0.05$ according to Fishers LSD set.

Table 3. Effects of the control, L1, C1 and NH$_4$VO$_3$ (AV) on relative water content (RWC) of mung bean at various concentrations.

| Concentration | Control     | L1          | C1          | AV          |
|---------------|-------------|-------------|-------------|-------------|
| 5 μM          | 81.29 ± 2.12c | 81.16 ± 1.95 | 81.53 ± 1.45c | 80.75 ± 2.01c |
| 10 μM         | 81.60 ± 2.45c | 81.29 ± 1.56 | 76.63 ± 1.58c | 68.29 ± 1.92 b |
| 20 μM         | 81.53 ± 2.74c | 79.92 ± 1.87c | 68.93 ± 1.94 b | 59.75 ± 1.86a |

Values are represented as mean ± SD (n = 3). Bars with different letters are significantly different at $p \leq 0.05$ according to Fishers LSD set.

In all the plants, electrolyte seepage from the membranes increased gradually with the increasing concentration of the Schiff base complex (C1) and NH$_4$VO$_3$ (AV) whereas in L1 there were no such changes in these parameters in relation to control. At 5 μM level, EL remained similar for both C1 and AV whereas 10 μM and 20 μM of AV caused considerable membrane leakage as compared to C1-treated plants. H$_2$O$_2$ accumulation was pretty similar in the Schiff base complex
(C1) and AV-treated plant leaves up to 10 µM whereas at 20 µM, there was greater H₂O₂ accumulation in AV-treated plants as compared to those treated with C1. Similar trends were also noticed for membrane lipids peroxidation (MDA). Greater impact of the Schiff base complex (C1) treatment on MDA accumulation was observed at 20 µM whereas in AV-treated plants significant induction was observed from 20 µM. Survival prospect of plants was measured in terms of cell viability. There were no significant changes in cell viability across all concentrations in L1-treated plants and even in C1- and AV-treated plants when given at 5 µM. But beyond that there was gradual drop of cell viability in both the Schiff base complex (C1) and AV-treated plants and this drop was found be slightly lesser in Schiff base complex-treated plants. Findings of the present study suggest that the ligand (L1) has neither any of positive or negative impact on the accumulation of stress indicators. So far the Schiff base complex (C1) and AV both elicited certain level of membrane injury and ROS accumulation. While at low concentrations (5 µM) both C1 and AV have similar impacts but at higher concentrations harmful effects of the Schiff base complex (C1) was comparatively lesser compared to those with AV regarding membrane injury and oxidative stress, i.e. it can impart comparatively better cell survival.

Figure 5. Effects of the control, L1, C1, and NH₄VO₃ (AV) on: (A) Cell viability; (B) electrolyte leakage; (C) H₂O₂ content, and (D) MDA content respectively of mung bean at various concentrations. Values are represented as mean ± SD (n = 3). Bars with different letters are significantly different at p ≤ 0.05 according to Fishers LSD set.
Total free amino acid and total soluble sugar

Amino acids play as key player in metal chelation by which plant detoxify or alleviate heavy metal stress. Therefore, it can be suggested that plants experiencing higher amount of vanadium-induced stress can accumulate more amount of free amino acid. Results revealed that although both the C1 and AV are responsible for alleviating free amino level in vigna seedlings but the accumulation is much higher in AV-treated plants. This signifies the more toxicity of AV than C1 for vigna plants (Figure 6). Total soluble sugar content was also estimated. Higher sugar content in cell symbolizes less stress. Results show that both the C1 and AV are responsible for the increase of soluble sugar content at low concentrations (5 μM). But at higher concentration (20 μM), the soluble sugar content decreases drastically for AV than C1-treated plants (Figure 7). This justifies that both the C1 and AV are beneficial for vigna plants at low concentrations but at higher concentrations AV become more toxic than C1.
Conclusions
Outcomes of the present experiment reveal that the Schiff base complex (C1) has less toxic effects than NH₄VO₃ on mung bean seedlings and it also provide better tolerance to vanadium toxicity. Though different stress marker and reactive oxygen species (ROS) accumulation were less and minimum pigment damage was noticed in the Schiff base complex (C1)-treated seedlings, the optimum positive impact largely depends on the dose. Beyond certain concentration, the complex (C1) may show inhibitory effects on the plants. Therefore, the present study revealed that heavy metal Schiff base complexes can be used as potential supplement to meet up micronutrient deficiency and at the same time such complexes can minimize the toxicity generated by application of different heavy metals.

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