CHAPTER II

2. Experimental Section

2.1. Source and purification of the chemicals used

All the chemicals used in various works embodied in this dissertation were of analytical reagent grade (A. R) and were used without further purification as procured from various commercial sources. The chemicals used are listed in the Table 2.1.

Table 2.1. Purity and source of the chemicals used.

Chemical compound	Mol. Formula	Purity	Source	CAS NO
Salicylaldehyde	$C_7H_6O_2$	99%	S. D. Fine Chemicals, India	90-02-08
***o*-vanillin**	$C_8H_8O_3$	99%	Sigma-Aldrich, Germany	148-53-8
1,2-ethylenediamine	$C_2H_8N_2$	99%	S. D. Fine Chemicals, India	107-15-3
1,2-phenylenediamine	$C_6H_8N_2$	99%	S. D. Fine Chemicals, India	95-54-5
Aniline	C_6H_7N	99%	S. D. Fine Chemicals, India	62-53-3
Cupric acetate monohydrate	$C_4H_8CuO_5$	99%	S. D. Fine Chemicals, India	6046-93-1
Zinc acetate dihydrate	$C_4H_{10}O_6Zn$	99%	S. D. Fine Chemicals, India	5970-45-6
Methanol	CH_3OH	99.8%	Sigma-Aldrich, Germany	67-56-1
Ethanol	C_2H_5OH	99%	Sigma-Aldrich, Germany	64-17-5
Vanadyl sulfate hydrate	$VOSO_4.xH_2O$	99%	Sigma-Aldrich, Germany	123334-20-3
Benzil	$C_{14}H_{10}O_2$	98%	Sigma-Aldrich, Germany	134-81-6
Benzidine	$C_{12}H_{12}N_2$	99%	Sigma-Aldrich, Germany	92-87-5

Dimethyl sulfoxide	C_2H_6OS	98%	S. D. Fine Chemicals, India	67-68-5
Acetonitrile	C_2H_3N	98%	S. D. Fine Chemicals, India	75-05-08

2.2. Experimental Methods

2.2.1. Different physico-chemical methods used

Different physico-chemical methods have been used to characterize the structure of Schiff base ligands and their transition metal complexes. A brief account of these methods is given below:

a) Elemental analysis: Elemental micro-analyses (C, H and N) of all the synthesized compounds were performed by using Perkin–Elmer (Model 240C) analyzer. The metal contents in the transition metal complexes were determined with the help of Atomic Absorption Spectrophotometer (Varian SpectrAA 50B as shown below in Fig 2.1) by using standard metal solutions (1000 ppm) procured from Sigma-Aldrich, Germany.

Fig 2.1. Atomic Absorption Spectrophotometer (Varian SpectrAA 50B).

b) Melting point: The melting point of the ligand and complexes were determined by open capillary method with the aid of a melting point apparatus as shown below in Fig 2.2.

Fig 2.2. Melting point apparatus.

c) Infrared spectra: Infrared spectra of the dried samples (as KBr pellets) were recorded on a Perkin-Elmer Spectrum FT-IR spectrometer (RX-1) operating in the region 4000 to 400 cm^{-1}. IR spectroscopic grade KBr from Sigma-Aldrich, Germany was used for preparing the pellets after drying the salt (KBr) in a drying pistol over anhydrous $CaCl_2$ for 24 hours and then kept in a vacuum desiccator over anhydrous $CaCl_2$ for using it during recording the IR spectra of the samples.

Fig 2.3. Perkin-Elmer Spectrum FT-IR spectrometer (RX-1).

d) Electronic spectra: Electronic spectra of the ligands and their transition metal complexes dissolved in a suitable solvent methanol were recorded on a Jasco V-530 double beam UV-VIS spectrophotometer (as shown in Fig 2.4) at ambient

Fig 2.4. Jasco V-530 double beam UV-VIS spectrophotometer.

temperature. The instrument was coupled with a thermostatic arrangement to maintain the temperature at 298.15 K. Quartz cells of 1 cm path length were used for holding the sample and the reference solvent during the spectral measurements.

e) Conductivity: Specific conductances were measured at (298.15 ± 0.01) K with a Systronic conductivity TDS-308 meter (shown in Fig 2.5). The conductance measurements were carried out by using a dip-type immersion conductivity cell (CD-10) with a cell constant of 1.0 ± 10% cm^{-1}. The instrument was standardized by using 0.1 (M) KCl aqueous solution. Measurements were made in a thermostatic water bath maintained at the experimental temperature with an accuracy of ± 0.01 K.

Fig 2.5. Systronic conductivity meter (TDS-308).

f) Mass measurements

Mass measurements were carried out on digital electronic analytical balance (Mettler Toledo, AG 285, Switzerland) as shown in Fig 2.6. This Digital balance can measure mass to a very high precision and accuracy. The mass measurements were accurate to ± 0.01 mg.

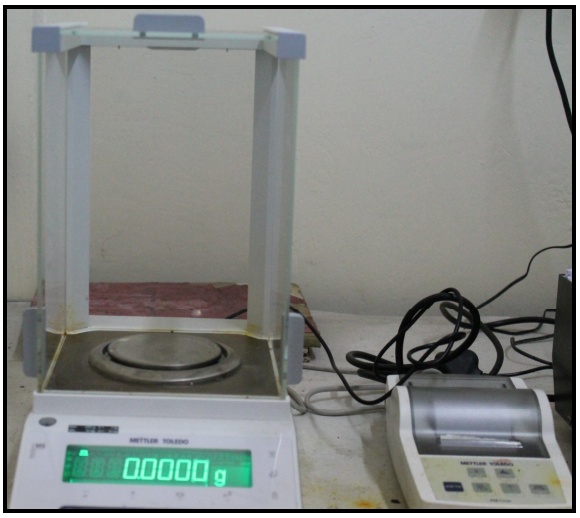

Fig 2.6. Digital electronic analytical balance (Mettler Toledo, AG 285).

2.2.2. Synthesis of the ligands and their transition metal complexes

a) Synthesis of N_2O_2 donor ligands and their Zn(II) complexes

Two different aldehydes, *viz*., salicylaldehyde and *o*-vanillin, each of purity level 99% were used without any further purification. $Zn(AcO)_2.2H_2O$, 1,2-ethylenediamine, 1,2-phenylenediamine and spectroscopic grade methanol were used for the synthesis of the ligands and their transition complexes. Slight alterations from standard or literature procedures were adapted during the preparation of both the ligands and their Zn(II) complexes.[1-3] Two aldehydes (Salicylaldehyde/*o*-vanillin) were alternatively treated in 2:1 molar ratio in MeOH with two corresponding amines (1,2-ethylenediamine/1,2-phenylenediamine) to prepare four ligands [**L1-L4**] with yields approximately 90-95%. The four prepared ligands were individually treated with $Zn(AcO)_2.2H_2O$ in MeOH in a molar ratio of 1:1 molar ratio and the respective mixtures were refluxed at temperatures 40-60 ^oC to obtain four corresponding Zn(II) complexes [**C1-C4**]. Characterization of these ligands and their Zn(II) complexes were done by elemental microanalyses and IR spectroscopic measurements. The

results were found to be more or less similar to those already reported in the literature.[3]

b) Synthesis of azo-functionalized Schiff base ligands and their Cu(II) complexes

0.1 mol of aniline was dissolved first in concentrated HCl. Next 8 g of analytical grade $NaNO_2$ (dissolved in bi-distilled water) was then added to the aniline solution drop wise with constant stirring for about 1 hour at 0 ^oC. A solution of 0.1 mol salicyladehyde, sodium carbonate (36 g) and water was added drop wise in the mixture with constant stirring. The reagents were allowed to react for ~5 hours at 0 ^oC. After the completion of the reaction light red colored precipitate of 4-(Benzeneazo)salicylaldehyde was filtered off and recrystalized from ethanol. The yield was approximately 80% and the melting point was found to be 118 ^oC. Treatment of two different amines (1,2-ethylenediamine and 1,2-phenylenediamine) with the azo part in 1:2 ratio in ethanol for ~3 hours at 80 ^oC to obtain two ligands [**L5** and **L6**]. These ligands were washed and recrystalized from ethanol and the yield was approximately 70%. The two prepared ligands were alternatively refluxed with $Cu(AcO)_2.H_2O$ in ethanol in 1:1 molar ratio for ~2 hours at 80 ^oC yielding two Cu complexes [**C5** and **C6**]. They were filtered, washed and then recrystalized with the yields ~70%. The two ligands **L5**, *i.e.*, {[N, N$^/$-bis[4-(benzeneazo) salicylaldehyde]ethylenediamine and **L6**, *i.e.*, [N, N$^/$-bis[4-(benzeneazo)salicylaldehyde]-*o*-phenylenediamine}and two complex **C5**, *i.e.*, {[N, N$^/$-bis[4-(benzeneazo) salicylaldehyde]ethylenediamine Copper (II) and **C6**, *i.e.*, [N, N$^/$-bis[4-(benzeneazo)salicylaldehyde]-o-phenylenediamine Copper(II)} were characterized by elemental microanalyses, IR spectroscopic measurements, conductometric measurements and electronic spectra. All results were more or less similar to those reported in the literature.[4]

c) Synthesis of tetradentate N_2O_2 donor Schiff base ligand and its vanadium(IV) complex

Ethanolic solution of benzidine (purity > 99%) was refluxed with benzil (purity 1 > 98%) in a round bottom flask in 1:2 molar ratio for ~4 hours at a temperature of 40-60 ^oC with constant stirring to get the olive green colored ligand **L7** [$C_{40}H_{28}N_2O_2$. $2H_2O$]. The ligand was filtered off, washed and recrystalized from ethanol. Then it was dried in a vacuum desiccator. To synthesize the vanadium(IV)

complex the ligand was further refluxed with an ethanolic solution of $VOSO_4.xH_2O$ for ~8 hours at 40-60 ^oC in a molar ratio of 1:1 with constant stirring. The complex **C7** obtained with a molecular formula of $C_{80}H_{56}N_4O_6V_2.5H_2O$ 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.[5]

2.2.3. Various morphological and biochemical parameters used

All the synthesized ligands and their transition metal complexes were applied to different plant materials. The various morphological and biochemical parameters monitored for the selected plants in different chapters are as follows:

a) Germination Index (GI)

Germination Index (GI) was calculated using following formula given by AOSA:[6]

$$GI = \left[\frac{a_1}{t_1}\right] + \cdots + \left[\frac{a_n}{t_n}\right]$$

where, a_1 , a_n, t_1 and t_n stand for the number of seeds germinated in first count, the number of seeds germinated in final (n^{th}) count, the days of first count and the days of final (n^{th}) count, respectively.

b) Germination percentage (GP)

Germination percentage was calculated 10 days after the germination by dividing the number of germinated seeds by total number of seeds in each pot, multiplied by hundred.[7]

$$GP = \frac{x}{y} \times 100$$

where, x and y stand for the total number of germinated seeds in each pot and the total number of seeds in each pot, respectively.

c) Seedling vigour index (SVI)

Seedling vigour index (SVI) was calculated according to Baki *et al.* as follows:[8]

$$SVI = L \times P$$

where, L is seedling length in cm and P is the germination percentage (GP).

d) Coefficient of velocity of germination (CVG)

Formula described by Maguire[9] was followed to calculate coefficient of velocity of germination. The formula is given below:

$$CVG = \frac{(G_1 + G_2 + \cdots + G_n)}{(1 \times G_1 + 2 \times G_2 + \cdots + n \times G_n)}$$

where, G_n is the number of germinated seeds after n^{th} day of germination.

e) Extraction and estimation of total soluble proteins

Ice cold 0.05 M sodium phosphate buffer solution (pH 7.2) was used for the extraction of total soluble protein from the seed, leaf and root tissues as per the method of Chakraborty *et al.*[10] Total soluble protein was estimated following by the method of Lowry *et al.*[11]

f) Extraction and estimation of chlorophyll

Chlorophyll from the leaves was extracted in 80% acetone in dark by following the method of Harborne by measuring the absorbance of the filtrate at 663 nm and 645 nm, respectively in a UV-VIS spectrophotometer.[12]

g) Extraction and estimation of carotenoids

Carotenoids from the leaves were extracted in 100% methanol following the method of Lichtenthaler[13] and the absorbance of the filtrate was immediately recoreded at 663, 645 and 480 nm in a UV-Visible spectrophotometer.

h) Extraction and estimation of antioxidative enzymes

For the extraction of antioxidative enzymes [peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APOX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6)], leaf tissues were homogenized in ice cold 50 mM sodium phosphate buffer solution (pH 7.2) with 1% (w/v) poly vinyl pyrrolidone and homogenate was centrifuged at 10000 rpm for ~20 minutes at -4 ^{o}C. The supernatant layer was used as crude enzyme extract. POX activity was assayed spectro-photometrically where the oxidation of *o*-dianisidine was observed following the method described by Chakraborty *et al.* with some modifications using UV-VIS spectrophotometer (Cole Parmer, USA). Method described by Asada and Takahashi[14] was followed for the assay of APOX activity, where quantity of ascorbate oxidized was measured spectrophotometrically. Catalase activity was measured by quantifying H_2O_2 broken down at 240 nm as described by Beers and Sizer.[15] Here 1 enzyme unit was interpreted as 1Δ absorbance per minute.

i) Extraction and estimation of total sugar

Total sugar from the leafs was extracted in 95% ethanol using the method of Harborne with minor modifications and Anthrone's method given by Plummer (1978) was followed for the estimation.[16]

j) Extraction and estimation of Leaf Zinc content

The Chickpea leaves were plucked off after 30 days from germination and were dried in a forced air oven at 70 ^{o}C to a constant weight and grounded to a finely divided powder. The formed dry samples (1 g) were combusted in a muffle furnace at 550 °C for ~8 hour. The ash samples were dissolved in 2(M) HCl following the method of Chapman and Pratt.[17] Zn content was estimated was with the aid of an atomic absorption spectrophotometer (Varian SpectrAA 50B) using standard Zn solutions for calibration.

k) Growth parameters

To determine fresh weight, the harvested plants were rinsed with de-ionized water and blotted on paper towels before being weighed. Dry matter yields of the seedlings were determined after drying the seedlings in an oven at 80 ºC to a constant weight. Relative water content (RWC) was measured according to the protocol described by Farooqui *et al.*[18]

l) Copper tolerance index

Copper tolerance index (TI) was calculated as the quotient of the dry weight of the plants grown under copper treated and control conditions according to the following formula:[19]

$$\text{TI}(\%) = \frac{\text{Dry weight of treated plants}}{\text{Dry weight of control plants}} \times 100$$

m) Electrolyte leakage

Ion leakage was measured as electrical conductivity (EC%) according to Yan *et al.*[20] The percentage of electrolyte leakage was calculated according to this formula:

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

where, C_1 and C_2 are the electrolyte conductivities measured before and after boiling, respectively.

n) Determination of free amino acids and proline

Free amino acids were detected according to the method of Lee and Takahashi.[21] Proline content was determined by following the ninhydrin method.[22]

o) Lipid peroxidation

It was measured as the content of malonyldialdehyde (MDA) using the thiobarbioturic method of Heath and Packer.[23]

p) H_2O_2 content

H_2O_2 levels in the leaves were estimated according to Jena and Choudhuri[24] with minor modifications. H_2O_2 levels were calculated by using extinction coefficient 0.28 μmol^{-1} cm^{-1}.

q) Cell viability

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 hours. Leaf samples were placed in 5% alcohol and kept for boiling till all the alcohol evaporated off. Thereafter the absorbance of the purple coloured extract was measured at 485 nm.[25]

r) 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 various concentration solutions of schiff base ligand (L7), Schiff base VO(II) complex (C7) and NH_4VO_3 (AV) for 6 days. Leaf discs floated in sterile distilled H_2O served as the experimental control The effects of different complexes on leaf discs were assessed on the basis of the phenotypic alteration especially leaf colour.

References

1) M. O´Conner, B. West, *Aust. J. Chem.* **20** (1967) 2077.
2) G. Batley, D. Graddon, *Aust. J. Chem.* **20**(1967) 885.
3) O. Kotova, S. Eliseeva, S. Averjushkin, L. Lepnev, A. Vaschenko, Y. Rogachev, *Russ. Chem. Bull.* **57**(2008) 1880.
4) J. N. Liu, B. W. Wu, B. Zhang, *Turk J Chem.* **30**(2006) 41.
5) A. Ahmed, S. BenGujji, *RASAYAN J. Chem.* **4**(2009) 781.
6) Association of Official Seed Analysis, *Seed Vigor Hand Testing Book*, Springfield, USA, 1983.
7) A. Cokkizgin, H. Cokkizgin, *Afr. J. Biotechnol.* **9** (2010) 8608.
8) A. Baki, J. Anderson, *Crop Sci.* **13**(1973) 630.
9) J. Maguire, *Crop Sci.* **2** (1962) 176.
10) U. Chakraborty, B. Chakraborty, M. Kapoor, *Fol. Microbiol.* **38** (1993) 491.
11) O. Lowry, N. Rosebrough, R. Randall, *J. Biol. Chem.***193** (1951) 265.

12) J. Harborne, *Phytochemical methods*, Chapman and Hall International ed, Toppan Company Limited, London, 1973.
13) H. Lichtenthaler, *Methods Enzymol.* **148** (1987) 350.
14) K. Asada, M. Takahashi, *Photoinhibition*. (1987) 227.
15) R. Beers, I. Sizer, *J. Biol. Chem.* **195** (1952) 133.
16) D. Plummer, *Introduction to Practical Biochemistry*, Tata McGraw-Hill, New York.
17) D. Chapman, P. Pratt, *Methods of Analysis for Soils, Plants, and Waters,* Division of Agricultural Sciences, University of California, Riverside, USA, 1961.
18) A. Farooqui, R. Kumar, S. Fatima*, J Plant Biol.* **27**(2000) 277.
19) A. F. Bálint, G. Kovács, J. Sutka*, Acta Biol Szegediensi.s* **46** (2002) 77.
20) B. Yan, Q. Dai, X. Liu, S. Huang, Z Wang, *Plant Soil*. **1972**(1996) 61.
21) Y. P. Lee, T. Takanashi, *Anal Biochem.* **14** (1966) 71.
22) L. S. Bates, R. P. Waldren, L. D. Tear, *Plant Soil*. **39** (1973) 205.
23) R. L. Heath, L. Packer, *Arch Biochem Biophys.* **125** (1968) 189.
24) S. Jena, M.A. Choudhuri, *Aquat Bot.* **12** (1981) 345.
25) H. Chen, Z. Shen, P. Li*, Crop. Sci.* **22**(1982) 719.
26) L. Fan, S. Zheng, X. Wang, *Plant Cell*. **9** (1997) 2183.