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Full Length Research Paper

The Effectiveness of Pre-Harvest Salicylic Acid Application on Physiological Traits in Lilium (Lilium longiflorum L.) Cut Flower

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Abstract. The aim of the study was to determine the effect of foliar application of salicylic acid (SA) on Lilium cut flower quality. At the stage of bud initiation, four concentrations (0, 50, 100 and 200 ppm) of SA were sprayed on shoot. After treatment, morphological and physiological characteristics were measured within 7 days. It is resulted that SA and Time have significant effect on flowering stem length (FSL), flowering stem diameter (FSD) and bud volume (BV) (P<0.01) and also the interaction between SA*Time has a significant effect on bud volume (P<0.05). According to the results, flowering stem length, flowering stem diameter and bud volume were significantly influenced by SA pretreatment and time. SA pretreatment at 50 ppm concentration had a positive effect on mentioned traits however at high concentration, especially at 200 ppm concentration, inhibitory effects were observed. There was an increasing trend in values for these traits over time and the highest values were obtained in the last day. Leaf chlorophyll and malondialdehyde content were not significantly affected by exogenous SA treatment hence promotion effects of SA on oxidative stress was not clear. Membrane stability of the younger leaves were greater than the older leaves furthermore the response of these leaves, against SA treatment, was more tolerable. Overall, the damaging effect of high dosages of SA is more on middle leaves and also this leaves showed more response to SA spray. In this case probably SA pre-harvest spray can done on the stem middle leaves and upper leaves and avoid of damaging effects of SA on other parts of plants specially flower bud induction or initiation. It is concluded that SA at 50ppm concentration improves morphological characteristics of Lilium cut flower so the flower quality increases in response to these modifications.

Key words: Lilium longiflorum, membrane stability, morphological characteristics, salicylic acid, bud size

1. INTRODUCTION

Short postharvest vase life of Lilium longiflorum is one of the most important problems in production of this valuable cut flower (Da Silva, 2003; Kader, 2003). To control this natural phenomenon, many studies are performed and some findings are improved vase life. Within those investigations the use of plant growth regulators such as GA4+7 plus benzyladenine (Catherine et al., 2001) and GA with 75 ppm concentration (Emami et al., 2011) has a remarkable position in reducing foliar chlorosis and increasing vase life of Lilium longiflorum, respectively. The contribution of cytokinin in flower longevity has been proved for dicotyledonous flowers (Mayak and Halevy, 1970) but the evidence on monocotyledonous flower is not clear.

Petals senescence commonly is accompanied by morphological, biochemical and biophysical deterioration which consists of declining protein content, increase in protease activity and decline in lipid fluidity in membranes (Arora et al., 2007). Furthermore, initiation of senescence in plant tissues is involved with reactive oxygen species (ROS) (Dhindsa et al., 1981). Activated oxygen species such as O2 and H2O2 and their interaction product, hydroxyl radical (OH), react with DNA, degrade proteins, lipids and nucleic acids leading to senescence (Arora et al., 2002). According to Mayak et al. (1983) superoxide anions (O2-) that are producing during senescence of carnation petal induce the degradation of phospholipids and the fatty acids released by this breakdown are then peroxidase, which in turn affects membrane permeability (Simon, 1974). The senescence of cut flowers is closely related to many factors that some factors such as variety, pre-harvest factors, food Supply, water supply (Sankat and Mujaffar, 1994) and mechanical damage are critical in life span of cut flowers. There are several documents based on using different treatments in postharvest for delaying senescence and enhancing cut flower vase life such as Rosa hybrida (Hajizadeh et al., 2012), Eustoma grandiflorum (kazemi et al., 2012) specially SA (kazemi et al., 2011; Geralloo and Ghasemnezhad, 2011). However plant growth situation during pre-harvest can effect on the quality of cut flowers about...
%30-70 (Halevy and Mayak, 1981) since, using of some treatments during pre-harvest is reasonable.

It has been found that (salicylic acid) SA plays a role during the plant response to abiotic stresses and regulates physiological and biochemical processes during the entire lifespan of the plant (Rivas-San Vicente and Plasencia, 2011). It has also improved in some vase life of cut flowers. For instance, Hatamzadeh et al. (2012) reported that Post-harvest treatment of SA with 150 mg/L concentration on cut gladiolus flowers caused an effective increasing on vase life, maintains higher spike fresh weight, antioxidant enzyme, stability of membrane and leading to delay in petal senescence.

Salicylic acid (SA) is a phenolic compound and natural constituent of plant (Raskin, 1992) which is recognized as an endogenous regulator in plants after the finding that it is involved in many plant physiological processes (Pancheva et al., 1996). Plants possess a well-defined enzymatic antioxidant defense system to protect themselves against these deleterious effects by scavenging ROS (Hatamzadeh et al., 2012). SA can neutralize oxidative stress by increasing in the activity of anti-oxidant enzymes (Tayeb et al., 2006). In recent years, several reports have identified the beneficial effects of salicylic acid in maintaining the quality of several species such as Rosa hybrida (Yongping et al., 2000) and Gerbera jamesonii (Yuping, 2009) cut flowers. For this reason, the present study aimed to investigate the effect of pre-harvest foliar application of salicylic acid (SA) on the growth, flowering and cut flower quality of Lilium longiflorum L. cv. Tressor. Furthermore, the effect of SA on oxidative stress in different parts of flower stem was studied.

2. MATERIALS AND METHODS

2.1. Sample preparation

A split plot experiment with two factors was performed in randomized complete block design with three replications to evaluate acid salicylic effects on growth and flowering performance of Lilium longiflorum L. The experiment was carried out in greenhouse. The temperature of the greenhouse was kept at 24±4 °C with 60-70 percent humidity. Lilium longiflorum L. cv. Tressor, flower F1 bulbs were planted to the experiment plots in a 8:8:1 (v/v) mixture of pit, perlite and sand. They were then irrigated with 1/2 Hoagland solution every 3 days. At the stage of bud initiation, four concentrations (0, 50, 100 and 200 ppm) of salicylic acid were sprayed on stem and sampling was done from three different parts of stem (lower, middle and upper). Distilled water was considered as control.

2.2. Analytical method

The following morphological and physiological characteristics were measured during 7 days after treatment:

Flowering characteristics:

Flowering Stems Length (FSL): Flowering stem length were measured by ruler from the basal point of stem until the beginning of inflorescence.

Flowering Stems Diameter (FSD): Diameter of flowering stem was evaluated by a coulis in the middle of stem during 1 week as daily recordings.

Number of Buds per inflorescence (NB): For this character the number of buds per each stem was counted.

Bud Volume (BV): For the measurement of bud volume the diameter and length of first bud on the inflorescence was measured in mm from the middle of a bud and through the bud from tip to end of bud, respectively on the first day before treatment until 6 day after treatment. Then the volume of bud calculated as an equation for volume of cylinder, approximately.

Chlorophyll Index: Chlorophyll index was measured by chlorophyll meter (SPAD-502, Minolta Co. Japan); this is presented by SPAD value. Average of 3 measurements from different spots of a single leave was considered.

Membrane stability index (MSI): For determination of membrane stability index (MSI), fresh leaf samples were cut into small discs of uniform size. Then samples were weighed and taken in test tubes containing 10 ml of double distilled water. These tubes were incubated at 40°C in a water bath for 30 minutes and electrical conductivity of the samples (C) was measured using conductivity bridge. The samples were transferred to the other test tubes and incubated at 100°C in the boiling water bath for 15 minutes and their electrical conductivity (EC) was measured as above. Membrane stability index was calculated and expressed in percentage using the formula (Premachandra et al., 1989), MSI= [1-(C1/C2)] x100.

Lipid peroxidation (MDA): Lipid peroxidation analysis was performed according to the method of Heath and Packer (1968) using 0.2 g of fresh leaf tissue from each treatment. 1 ml MDA extract was added to 4 ml trichloroacetic acid containing 0.5% thiobarbituric acid. The solution was heated at 95 °C for 30 min and then quickly cooled in running water. The solution was centrifuged at 10000 g for 10 min. The absorbance of the supernatant was measured at 532 and 600 nm. The concentration of MDA was calculated by subtracting absorbance at 600 nm from absorbance at 532 nm, and expressed as mg MDA g fresh weight (= 155 mM⁻¹ cm⁻¹).
2.3. Statistical analysis

The data were analyzed for variance using ANOVA procedure in SAS software version 9.1 (SAS Institute, Cary, North Carolina, USA). The differences between means were compared by Duncan’s multiple range tests. Statistical significance was considered at p<0.05. Note: in this study effect of main plots was not significant hence the analysis of data were reported in factorial.

3. RESULTS AND DISCUSSIONS

3.1. Effect of SA on flowering characteristics

According to table 1 it is resulted that SA and Time have significant effect on flowering stem length (FSL), flowering stem diameter (FSD) and bud volume (BV) and also the interaction between SA*Time has a significant effect on bud volume.

3.2. SA and Bud Volume (BV)

Salicylic acid treatment at 50 ppm concentration did not increase bud volume significantly in comparison to the control. While with increasing SA to higher concentrations BV was decreased significantly (Table 2) and the highest inhibition effect obtained by 200 ppm concentration (Tab 2). Our findings are agree with Sabzi et al. (2012) which suggested 1mM SA similar to control had the most effect on rose flower diameter compare to 2 mM SA. According to Table 1 the interaction between SA and Time on Bud Volume (BV) was significantly different. As illustrated in figure 1 the volume of Lilium buds increased along with time as bud volume at before treatment until 6th day increased from 20.973 mm to 53.799 mm.

Table 1: Analysis of variance for flowering characteristics

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>FSL</th>
<th>FSD</th>
<th>NB</th>
<th>BV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>3</td>
<td>92.24**</td>
<td>1.23**</td>
<td>1.04</td>
<td>201.79**</td>
</tr>
<tr>
<td>Time</td>
<td>6</td>
<td>67.65**</td>
<td>0.87**</td>
<td>1.05</td>
<td>1791.04**</td>
</tr>
<tr>
<td>SA*Time</td>
<td>18</td>
<td>4.85</td>
<td>0.04</td>
<td>0.84</td>
<td>56.25*</td>
</tr>
<tr>
<td>E</td>
<td>34</td>
<td>13.5045</td>
<td>0.1027</td>
<td>1.4521</td>
<td>12.9144</td>
</tr>
</tbody>
</table>

** significant at p<0.01, * significant at p<0.05, SA: Salicylic acid, BV: Bud Volume, FSD: Flowering stem diameter, FSL: Flowering stem length, NB: Number of buds

Table 2: Means comparison of salicylic acid treatments on flowering characteristics

<table>
<thead>
<tr>
<th>Treatment Salicylic acid (ppm)</th>
<th>Bud Volume (mm)</th>
<th>Flowering stem Length(cm)</th>
<th>Flowering stem Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38.785a</td>
<td>57.530c</td>
<td>9.503b</td>
</tr>
<tr>
<td>50</td>
<td>38.945a</td>
<td>60.529a</td>
<td>10.006a</td>
</tr>
<tr>
<td>100</td>
<td>34.212b</td>
<td>59.281ab</td>
<td>9.623b</td>
</tr>
<tr>
<td>200</td>
<td>32.931b</td>
<td>55.696c</td>
<td>9.485b</td>
</tr>
</tbody>
</table>

3.3. SA and flowering stem diameter (FSD)

Salicylic acid at 50 ppm concentration affected flowering stem diameter positively but other concentrations of SA had no significant differences with control (Table 2). Results obtained from figure 1 showed that there is an increasing trend in flowering stem diameter from start till 4th day but there was no change in stem diameter after that. It is suggested that foliar SA applications on Saintpaulia significantly improved some plant characteristics such as the number of leaves, rosette diameter and the number of days from potting to anthesis (Jabbarzadeh et al., 2009). It seems that positive effect of SA on growth parameters are attributed to enhanced CO₂ assimilation, chlorophyll concentration and photosynthetic rate (Karlidag, 2009).
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3.4. SA and flowering stem length (FSL)

According to Table 2 it is resulting that there is a significant difference between SA at level of 50 ppm and 200 ppm as well as control in flowering stem length, thus Liliums which are treated with 50 ppm SA have the most stem length in comparison with others. The same results obtained in Gladiolus which sprayed with 50 ppm SA by Ram et al. (2012). On the other hand, the inhibitory effect of SA on stem length is clearer at level of 200 ppm. The stimulatory effect of SA has been demonstrated before by Handro et al. (1997) who reported that using of SA at (0.5-1µM) in medium culture of Ullucus tuberosus caused to more elongated axillary shoots than controls. As shown in figure 1 flowering stem length had the increasing trend until 3th day bud no significant difference in flowering stem length was observed after that. As Salicylic acid is a growth promoting chemical (Ram et al., 2012) has a favorable effect on growth parameters and it seems that accelerates the cell divisions and cell elongation in the apical portion of stem.

3.5. Effect of SA on physiological parameters

Besides the effect SA on flowering parameters other observations was carried out about the role of SA in preserving quality of leaves and preventing of lipid peroxidation among tree different parts of the stem leaves (lower, middle and upper). According to table 3 the effect of leaf position (LP) on Chlorophyll (p<0.01) and the interaction between SA×LP on membrane stability Index (MSI) (p<0.05) were significant. Means comparison for chlorophyll content showed that the amount of chlorophyll in leaves harvested from different part of stem is significantly different. The lower, middle and upper leaves chlorophyll content were 43.93, 59.60 and 62.91 (µg cm⁻²), respectively. Also there was no significant difference in Malondialdehyde (MDA) between 3 levels of leaf position and also different concentrations of SA treatments (Table 3).

Table 3: Analysis of variance for physiological parameters

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Chl</th>
<th>MDA</th>
<th>MSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>3</td>
<td>14.89</td>
<td>0.132</td>
<td>123.46</td>
</tr>
<tr>
<td>LP</td>
<td>2</td>
<td>112.42**</td>
<td>0.060</td>
<td>179.52</td>
</tr>
<tr>
<td>SA×LP</td>
<td>6</td>
<td>44.20</td>
<td>0.205</td>
<td>162.46*</td>
</tr>
<tr>
<td>E</td>
<td>24</td>
<td>444.86</td>
<td>0.0849</td>
<td>59.6</td>
</tr>
</tbody>
</table>

** significant at p<0.01, * significant at p<0.05SA: salicylic acid, LP: leaf position, Chl: Chlorophyll content, MDA: Malondialdehyde, Membrane stability index: MSI

Results from table 3 showed that the interaction between SA×LP was significantly different (p<0.05) on membrane stability index. According to figure 2 the least and highest membrane stability index were related to lower stem leaves and stem upper leaves, respectively. It appears that deterioration effects of SA on older leaves are greater that the younger leaves and with increasing SA concentration those effects are severe (Fig 2).
Fig. 2: Means comparison of SA×LP on membrane stability index; lower leaves (1), middle leaves (2) and upper leaves (3)

It seems that the effect of SA at high dosages not only is advantage but also is very injurious. However upper stem leaves seems to have constant membrane stability for different applications of SA concentrations. It is suggested that juvenile leaves have less susceptibility to different levels of SA treatment. At middle leaves which seems to be more active in photo assimilation there was no significant difference between control and SA 50 ppm and 100 ppm treatments in MSI but an increasing trend in membrane stability index was observed with increasing level of SA to 50 and 100 ppm but not for 200 ppm. Overall, the damaging effect of high dosages of SA is more on middle leaves and also this leaves showed more response to SA spray. In this case probably SA pre-harvest spray can done on the stem meddle leaves and upper leaves and avoid of damaging effects of SA on other parts of plants specially flower bud induction or initiation.

4. CONCLUSIONS

It is clear that SA has the important role in the control of several physiological and biochemical processes in plants especially flower-inducing but this phenomenon can occur in combination with other plants growth regulators (e.g. gibberellins) and also it is related to day length strongly. So, having no effect on bud number in our findings is acceptable and needs to be studied along with other factors. On the other hand it seems that the inhibitory effect of SA on ethylene production resulting in stimulation of growth and regenerative capacity. Salicylic acid applied to the foliage of intact plants induced positive effects on the bio-productivity of horticultural and ornamental plants. Moreover, in order to get a desired effect it was observed that lower concentrations of salicylic acid are needed.

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- The Effectiveness of Pre-Harvest Salicylic Acid Application on Physiological Traits In Lilium (Lilium longiflorum L.)


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Full Length Research Paper

Studying the Relationship between Acyl-CoA-Binding Protein 2 and Lysophospholipase 2 in Arabidopsis thaliana and Their Importance in Recycling of Cadmium

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Abstract. A major environmental concern due to dispersal of industrial and urban wastes generated by human activities is the contamination of soil. A wide range of inorganic and organic compounds cause contamination, these include heavy metals, combustible and putrificable substances, hazardous wastes, explosives and petroleum products. Major component of inorganic contaminates are heavy metals. Phytoremediation is a group of technologies that used by plants to remove, degrade or immobilize environmental toxins. This technology largely focused on the use of plants for accelerating degradation of organic contaminants. But in addition to use of plant for soil remediation, hyper accumulator plants can be used for recycling of metals from soil. Hyper accumulator plant express special proteins that help them to tolerate the high concentration of metals. In this experiment we discussed the relationship between acyl-CoA-binding protein 2 and lysophospholipase 2 and showed why this two proteins work together.

Key words: phytoremediation, immobilize, toxin, organic contaminants

1. INTRODUCTION

Evolution of human life led to the development of technologies and appearance of secure sciences (Bennett et al., 2003). Global development, created treatable issues especially in the area of environment protection and natural resources. Since the industrial revolution, soil contamination by toxic metals has been severely increased. According to the Ryagys article 90% of metal diffusion happened by artificial sources (Ikuhoria et al., 2000). Trace metals, including those defined as “heavy”, arise from industrial and mining activities discharge into coastal waters and estuaries at many sites. Any metallic element that has a high density and is toxic is called heavy metal. These anthropologically derived inputs can accumulate in local sediments and invertebrates living on or in food, and the rate of accumulation varies widely between species and heavy metal concentration found in “clean” conditions (Harris and Santos, 2000).

Soil and water contaminated with metals include a major environmental and human health problem that is still in need of an effective technological solution. Nonradioactive As, Cd, Cu, Hg, Pb and Zn and radioactive Sr, Cs and U are the most environmentally important metallic pollutants. Microbial bioremediation has been somewhat successful for the degradation of certain organic contaminants, but is ineffective at addressing the challenge of toxic metal contamination, particularly in soil (Dudka and Miller, 1999). Today it is clear that human activities lead to increasing the metal pollutant in soil and water (Nriagu, 1996). Mining and smelting operations are important causes of heavy metal contamination in the environment due to activities such as mineral excavation, ore transportation, smelting and refining, and disposal of the tailings and waste waters around mines (Dudka and Adriano, 1997; Navarro et al., 2008). A lot number of chemical materials, heavy metals and other industrial wastes discharged in coastal regions. This toxic materials spread in environment and thought the food chain lead to toxic effects on human and animals (Dembitsky, 2003; Manohar et al., 2006).

Ordinarily the soil is a place for disposal of many heavy metals. Today the methods for reducing metal pollutants in soil are expensive method and damage to environment. So finding low cost methods for disposing of heavy metals (BIO-WISE, 2003; Aboulroos et al., 2006) Metal-binding proteins play critical catalytic, regulatory and structural roles in the cells. (Formigari et al., 2007) Their Identification and characterization can contribute toward a better use of...
them in metal pollutant remediation. ACBPs are implicated in acyl-CoA trafficking in many eukaryotes and some prokaryotes. Six genes encode proteins designated as AtACBP1–AtACBP6 in the Arabidopsis thaliana ACBP family. These ACBPs are conserved in the acylCoA-binding domain. In Arabidopsis, the ACBP family consists of six members (AtACBP1–AtACBP6) with AtACBP6 being the smallest (10.4 kDa) and AtACBP4 the largest (73.1 kDa).

As in stress full conditions like metal pollutant environments the coding of Acyl-CoA binding protein increased we try to understand the effect of this change (Blanvillain et al., 2009). The use of plants to remove heavy metals from contaminated soils can be also applied as a tool for extracting metals, especially trace metals from soil. Today Phytostabilisation is mostly used for the remediation of soil, sediment and sleges (Mueller et al., 1999) and depends on roots ability to limit contaminant mobility and bioavailability in the soil (Berti et al., 2000). It is a good approach to remove the contamination primarily from soil and isolate it, without destroying the soil structure and fertility. The removed heavy metal can be recycled from the contaminated plant biomass (Brooks et al., 1998). Factors such as growth rate, element selectivity, resistance to disease, method of harvesting, are also important (Cunningham et al., 1996). However slow growth, shallow root system, small biomass production, final disposal limit the use of hyper accumulator species (Brooks et al., 1994). In this study we tried to understand the mechanism of cadmium tolerance in plant Arabidopsis thaliana. By finding the mechanisms of tolerance in hyper accumulator plants and strengthen this pathways can be hoped to produce more resistant plant and overcame the problems that mentioned.

Fig. 1: Amino acid sequence of Acyl-CoA-binding domain-containing protein 2

1.1. Heavy metals

Heavy metals are the main mineral contaminants and due to the use of sludge or compost from urban excrement, pesticides and fertilizers, heavy metals published in the environment and pollutions are causing significant portion of earth (Halim and Conte, 2003) Although metals are naturally present in the Earth’s crust and many of them are essential for cellular processes but all metals are toxic in high concentrations (Xiaoe Yang et al., 2005). Heavy metals in soil can be a danger for the health of human and animals. As, Cd, Cr, Cu, Pb, Mg, Ni, Se, Ag and Zn are the metals that have this bad effects (McIntyre, 2003).Other metals that have such effects are Al, Cs, Co, Mn and Sr. All plants can absorb and accumulate essential metals. Plants for growth need to several concentrations of any types of metals (Long, 2002). This ability of plants let them to accumulate other non-essential metals. When the concentration of metals in plants cells become more than their threshold, it inhibit the normal function of cytoplasmic enzymes and causes cell toxicity and also through transporting nutrients in place of cation exchange cause indirect toxicity (Djingova and Kuleff, 2002).

Fig. 2: 3 dimensional structure of Acyl-CoA-binding domain-containing protein 2
2. MATERIALS AND METHODS

Acyl-CoA binding protein sequence was obtained from NCBI – GenBank (figure 1), Protein Data Bank (PDB) and the 3D structure of lysophosphatidylcholine obtained from chemspider website (figure 2). In order to design the 3D structure for Acyl-CoA binding protein first of all PSI-BLAST performed and the 3D structure of homologues of acyl-CoA binding protein obtained. For designing 3D structure Easy modeler software was used. This software according to 3D structure of homologues proteins released a new 3D structure for unknown protein. Phyre2, SAM-T08 and m4t servers also used for predicting 3D structure of Acyl-CoA binding protein and the best model with the lowest Q-mean score accepted. Energy minimization performed using UCSF chimera candidate version1.5.3. During minimization step update interval was 10 and step size was 0.02. After that, this protein was docked with lysophosphatidylcholine using Molegro software. Molegro Virtual Docker is an integrated platform for predicting protein - ligand interactions. Molegro Virtual Docker handles all aspects of the docking process from preparation of the molecules to determination of the potential binding sites of the target protein, and prediction of the binding modes of the ligands (http://www.molegro.com/trial.php).

3. RESULTS AND DISCUSSIONS

Metalloproteins are a large and diverse class of proteins which bind one or more metal ions in their native con- formation (1). A metal binding site typically consists of an ion bound to one or more protein residues (called ligands). Among the 20 amino acids, the four most common ligands are cysteine (C), histidine (H), aspartic acid (D), and glutamic acid (E). Highly conserved residues are more likely to be involved in the coordination of a metal ion, although in the case of cysteines, conservation is also often associated with the presence of a disulfide bridge (a covalent bond between the sulfur atoms of two cysteines). In this study we tried to understand if there is any interaction between acyl-CoA binding protein and lysophosphatidylcholine. The secondary structure of acyl-CoA binding protein constructed with Easy Modeler software and showed with its three cofactors(SO4) in figure 1. this protein docked by Molegro software with lysophosphatidylcholine and

The results of this software indicate that acyl-CoA binding protein has high affinity to lysophosphatidylcholine. The moldocke scores for 10 times Molegro running were all lower than -80 kJ/mol. As this docking studies have low moldocke scores, so it is clear that there is a direct relationship between cadmium tolerance and acyl-CoA binding protein expression.

5. CONCLUSION

There are many proteins including P1B-type heavy metal ATPases, ABC transporter, phytochelatins, methallothioneins and oxidative stress-related proteins have been associated with heavy metal stress (Wei Gao et al., 2010) Some Heavy metals including Cu(II), iron [Fe(III)], nickel [Ni(II)], Cd(II) and Zn(II) are known to induce oxidative stress at high concentrations(Guerrero et al., 2006). In vitro observations showed that in response to metal stress the expression levels of lysophospholipase increased.
Also we know that Arabidopsis thaliana lysophospholipase 2 (lysoPL2) binds acyl-CoA-binding protein 2 (ACBP2) to mediate cadmium [Cd(II)] tolerance (Larsen et al., 2006). Our results show that acyl-CoA binding protein has high affinity to lysophosphatidylcholine with average energy - 83Kj/mol. So we discussed that in order to decrease the digestive effect of increase in lysophospholipase level on cell membrane, the acyl-CoA binding protein attached to lysophospholipase 2 and by saturating lysophospholipase activation site moderate its digestive effects and increased Cd tolerance in Arabidopsis thaliana.

Fig. 3: The secondary structure of acyl-CoA binding protein and lysophosphatidylcholine (yellow)

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Gavanji et al.

Studying the Relationship between Acyl-CoA-Binding Protein 2 and Lysophospholipase 2 in *Arabidopsis thaliana* and Their Importance in Recycling of Cadmium

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Methyl Violet Removal from Synthetic Wastewater by Liquid-Liquid Extraction using Vegetable Oils as Solvent

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Abstract. A laboratory study on methyl violet removal from aqueous solutions by liquid–liquid extraction system has been carried out, using crude palm oil and soy bean oil as solvent. The concentration of methyl violet has been studied in the range of 100 mg/L to 200 mg/L. The efficiency of dye extraction increased when two extractant di-2-ethylhexyl phosphoric acid and tributhyl phosphate were used as carrier. Under optimized conditions, 82% to 89% dye removal in 5 minutes rapid mix followed by 60 minutes slow mixing was achieved. The aqueous to organic phase volume ratio (A/O) was fixed at 1, pH varied from 1-6 and extraction study was carried out at [TBP]: 200mM and [D2EHPA]: 75mM.

Key words: Liquid–Liquid extraction, Methyl violet, D2EHPA, TBP

1. INTRODUCTION

Synthetic dyes are essential refractory organic compounds and are often found in the environment as a result of their wide industrial usage (Dâas and Hamdaoui, 2010). Dyes are broadly used in numerous industries such as textile, paper, artificial manufacturing, cosmetics and leather, for tinting and colouring the products. The discharge of dye wastewater from these industries contains harmful and toxic materials and is considered as a potential source of pollutants in environment which may accumulate to a toxic concentration and cause serious problems (Muthuraman and Teng, 2009). Based on above mentioned facts, the dye removal from water and as the main source, industrial wastewater is very important and essential treatment is somehow required before contaminated industrial waste discharge.

Typical and popular wastewater treatment methods are not very effective for dye removal, particularly because the dye stability against light oxidation and also being resistant to aerobic bio-oxidation (Poots et al., 1976).

Liquid–liquid extraction (LLE) is considered as a treatment method for purification of various compounds in mixtures (Garg et al., 2008; Njau et al., 2000; Cheng 2000; Thornton 1992). Extraction is a diffusion separation process of moving analyses from the matrix to a physically separate location where further processing and analysis occur. LLE is based on the distribution of a solute in a specific ratio between two immiscible solvents (Robbins and Cusack, 1997; Lee et al., 2009).

However, typical and popular organic solvents used in LLE as a diluent is considered hazardous, recalcitrant and toxic to environment and researchers are trying to find better and non-toxic diluent which are environmentally friendly and less harmful, such as vegetable oils.

In this research, the LLE efficiency of a cationic dye namely methyl violet (MV) using di-2-ethylhexyl phosphoric acid (D2EHPA) and tributhyl phosphate (TBP) prepared in crude palm and soy bean oil as organic phase was studied. The effect of pH, extractant type and different vegetable oils were investigated and the process was optimized by factorial design.

2. EXPERIMENTAL DESIGN AND METHOD

2.1. Reagents

The required stock solutions of MV was prepared by dissolving the certain amount of the dye in distilled water and made up to 1000 mL. MV was purchased from Merck. Other chemicals such as NaOH, H2SO4 were obtained from R&M.

2.2. Apparatus and Measurements

For pH measurement of aqueous phase, pH meter (HACH, Germany) was used. Dye concentration was measured and analysed by Spekol 1200, Germany Spectrophotometer. An overhead stirrer (IKDK, Germany) was used for solutions mixing and agitation.
2.3. Procedure

Solvent extraction experiments were undertaken at room temperature. H₂SO₄ and NaOH were used for pH adjustment. Aqueous phase containing dye (100 mg/L, 25 mL) and the organic phase containing vegetable oil and carrier (25 mL) were mixed for 5 minutes at 120 rpm (rapid mixing) followed by one hour slow mixing at 50 rpm. Aqueous and organic phases were introduced in a separating funnel to separate. After certain time of settlement, the samples were taken from the lower phase of the separated funnel (aqueous phase), filtered through filter paper and the dye concentration was measured by UV-Spectrophotometer.

Dye removal percentage (extraction percentage) was calculated based on equation below:

\[ E = \frac{[\text{dye}]_{\text{org}} - [\text{dye}]_{\text{aq}}}{[\text{dye}]_{\text{aq}}} \times 100 \]  

Where \([\text{dye}]_{\text{aq}}\): initial dye concentration in the aqueous phase (mg/L); \([\text{dye}]_{\text{org}}\): dye concentration of aqueous phase after extraction (mg/L).

For preliminary studies, in each experiment, 25ml of aqueous solution with 100 mg/L dye concentration was poured into a 250 ml beaker. A ratio of 1 was selected for aqueous to organic phase ratio. According to the literature, if the aqueous to organic phase ratio is beyond 1/1, then further increase in the volume of internal aqueous solution will have a negative effect on both the rate and efficiency of extraction process (Días and Hamdaoui, 2010). Based on previous studies, this is due to the fact that in case higher aqueous to organic phase ratio beyond 1, increase of the emulsion viscosity and also an increase of the diameter of internal droplets will occur (Dj enouhat et al., 2008; Chiha et al., 2006). On the other hand, the bigger droplets diameter will have a direct negative effect on interfacial contact area between the two phases and as a consequence, this will drop the extraction efficiency. In addition to, the other difficulty for higher volume ratios is due to the volume of membrane solution which is not enough for infolding the stripping solution (Juang and Lin, 2004).

All the experiments were run base on statistical design of experiments in duplicate and analytical parameters were performed in triplicate for each run. The pH was adjusted to desired value (1-6). The organic phase was prepared for three types of carrier (TBP and D2EHPA and a mixture of carriers) and introduced into two different vegetable oils (crude palm oil and crude soy bean oil) as solvent.

3. RESULTS AND DISCUSSIONS

3.1. VARIABLES, RESPONSES, DESIGN of EXPERIMENTS and DATA ANALYSIS

Two major variables were chosen based on the literature review most effective parameters. Among various effective variables such as stirring time, solvent type, different organic carriers, temperature etc. solvent, carriers and initial pH were chosen as main and most effective variables. Then a factorial design was applied for optimization process, including more than 100 different and various runs (by 5 different designs) in order to get the best applicable design of experiments. Then after, analysis of variance was applied for data analysis and results were examined to find the optimum condition for dye removal.

3.1. Statistical Analysis and Optimization

Factorial designs are the way to investigate the relationships among several factors, each at more than one level. Factorial designs produce efficient experiments. Each observation provides information about all of the factors and enables the researcher to look at responses to one factor at different levels of another factor in the same experiment. Furthermore, factorial designs provide information whether the factors act on the experimental units independently of one another or they don’t act independently indicating to the presence of interaction between the factors. Factorial designs allow the researcher to study:

1- The response variation created by a verify in the level of the factor which is called the main effect.

2- The alteration in response between the levels of one factor and all levels of another factor which is called the interaction (Myer and Montgomery, 2004; Tabachnick and Fidell, 2007).

General case of factorial design where there are a levels of factor A, b levels of factor B, c levels of factor C, and so on, in this case there will be a, b, c… n total number of observations (n represents the number of replication). In applying factorial design it is compulsory to use at least two replicates in order to determine a sum of squares due to error if all interactions are included in the model.

Special cases of factorial designs are the three-level factorial designs, where each factor at three levels, and two-level factorial designs, where each factor at two levels.

Three major factors were selected for the factorial design and process optimization. pH (4-6), carrier ([TBP]: 200 mM ; [D2EHPA]: 75 mM and mixture of both carriers) and vegetable oil (crude palm oil and soy bean oil).
The selected range for each variable is chosen based on literature review and also considering that the novelty and new contribution be satisfied. Based in literature, the highest dye removal by LLE is achieved in acidic range of pH, however, in preliminary study both acidic and alkaline regions were gone under examination (Muthuraman and Teng, 2009; Madaeni et al. 2011).

The results of analysis of variance ANOVA for color removal showed that the main effect of selected factors on dye removal was significant individually; meaning that each selected factor, pH, carrier and vegetable oil shows a direct and significant effect can remove the dye. However, the interaction between selected pH values and each carrier or mixture of carriers and the interaction between pH and vegetable oils did not show any significant affect. But results of NOVA reveals that interaction between the carriers and vegetable oil shows a significant impact on the dye removal.

### 3.2. Results

The results of analysis of variance (ANOVA) for dye removal are given in Table 1. The analysis of variance revealed that a first-order model adequately fitted the experimental data. The linear effect of pH (A), carriers (B) and vegetable oils (C) were significant (p-values < 0.05). The interaction between selected pH value (4-6) and two other factors were not significant, while the interaction between carriers and vegetable oils was significant, with $R^2$: 0.9858, Adj $R^2$: 0.9766.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>DF</th>
<th>Mean Square</th>
<th>F-value</th>
<th>Prob&gt;F</th>
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<td>9</td>
<td>542.05</td>
<td>107.76</td>
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</tr>
<tr>
<td>A</td>
<td>25.22</td>
<td>1</td>
<td>25.22</td>
<td>5.01</td>
<td>0.0419</td>
</tr>
<tr>
<td>B</td>
<td>4385.29</td>
<td>2</td>
<td>2192.64</td>
<td>435.91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C</td>
<td>265.33</td>
<td>1</td>
<td>265.33</td>
<td>52.75</td>
<td>&lt;0.0001</td>
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<td>1.45</td>
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<tr>
<td>AC</td>
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<td>0.000</td>
<td>0.000</td>
<td>1.000</td>
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<tr>
<td>BC</td>
<td>199.69</td>
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<td>99.85</td>
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<td>2</td>
<td>6.86</td>
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<td>12</td>
<td></td>
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<tr>
<td>Total</td>
<td>4948.84</td>
<td>23</td>
<td></td>
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</tbody>
</table>

Regression model was fitted to the data and a model was built, the model shows only significant terms in coded form includes the main effect for all factors and the interaction between carrier and vegetable oil is given in Eq.2

\[
\]

Where A, B and C represent the pH, carrier and the vegetable oil, respectively.

A positive sign for the regression coefficient in the fitted model indicates the ability of the factor to increase the response, while the negative sign indicated the ability of a factor to decrease the response.

The results of various combinations of different factors are shown in Table 2. The highest dye removal (89.1%) was achieved at pH 4, when a mixture of 200mM TBP and 75mM D2EHPA were used as the carrier and crude palm oil was used as the solvent.

The regression model obtained for dye removal is satisfactory since the value of the coefficient of determination $R^2$ is high and close to 1. The value of $R^2$ for dye removal model is 0.9858 and the value for adjusted $R^2$ is 0.9766. Interaction plot for significant interaction between carriers and vegetable oil is given in Fig. below showing the behaviour of these factors.
Based on preliminary results, a minor fluctuation in pH was observed after the extraction process was completed. This further increasing or decreasing pH in the feed phase which led to lower extraction efficiency could be due to the reducing fraction of MV molecules in the cationic form which exists in the aqueous solution. The observed low extraction efficiency of MV at pH < 4 is caused by declining the driving force of the extraction process. Similar behaviour was observed for peptide extraction as reported by Drapala, 2004.

Figure 1. shows the interaction effect between the pH and the carriers. Based on this and ANOVA analysis, if the pH value varies from 4-6, the carrier selection (D2EHPA, TBP or a mixture of both carriers) does not show any significant effect. The results reveal that at 100 mg/L of MV and 200 mM TBP, 55.4% removal was achieved at pH 4 and 50% removal was achieved at pH 6, using palm oil as diluent. For the same reason, when the pH was fixed at 4, 100 mg/L of initial MV at 200 mM TBP, showed 50.6% dye removal, and at pH 6, 50.5% dye removal was achieved.

Figure 2. shows the interaction effect between the carriers and diluents. The interaction was significant and the highest removal occurred when the palm oil used as the diluent and a mixture of TBP and D2EHPA used as carrier.

The highest removal in this combination (89.1%) dye removal was for pH 4.0, 100 mg/L initial concentration of MV, 75 mM of D2EHPA and 200 mM of TBP, using palm oil as diluent. For soy bean oil, the highest dye removal (78.9%) achieved for pH 4.0, 75 mM of D2EHPA and 200 mM of TBP. These results show that significant interaction is due to the presence of TBP and D2EHPA together as carrier.

Transport of MV using D2EHPA and TBP carrier, follows the same method in which metallic cations (Cote and Bauer, 1998) in a facilitated counter-coupled transport obeys, according to the equation represented in the equilibrium below:

\[ \text{MV}^+ (\text{org}) + \text{HA} (\text{aq}) \leftrightarrow \text{MVA} (\text{org}) + \text{H}^+ (\text{aq}) \]

where MV+ is methyl violet (cationic dye) and A is ligand. It should be mentioned that in reality, the complexes do not encompass the molecular form (HA) of the monomeric carrier in solution, which is totally in contrast to their analog structure containing one or more oxygen atoms (Sole and Hiskey, 1995).

Figure 3 shows the extraction process in organic and aqueous interface. HA represents the D2EHPA or

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**Table 2: Results of factorial design of experiments**

<table>
<thead>
<tr>
<th>Run</th>
<th>Carrier</th>
<th>Solvent (crude oil)</th>
<th>Initial pH</th>
<th>Final pH</th>
<th>Initial color</th>
<th>Final color</th>
<th>Predicted Value (%)</th>
<th>Actual Value (%)</th>
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<td>1</td>
<td>TBP</td>
<td>Soya oil</td>
<td>4</td>
<td>3.89</td>
<td>328</td>
<td>162</td>
<td>52.35</td>
<td>50.6</td>
</tr>
<tr>
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<td>TBP</td>
<td>Soya oil</td>
<td>4</td>
<td>3.77</td>
<td>192</td>
<td>92</td>
<td>52.35</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>TBP</td>
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<td>5.47</td>
<td>328</td>
<td>151</td>
<td>51.15</td>
<td>53.9</td>
</tr>
<tr>
<td>4</td>
<td>TBP</td>
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<td>5.5</td>
<td>192</td>
<td>95</td>
<td>51.15</td>
<td>50.5</td>
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<tr>
<td>5</td>
<td>D2</td>
<td>Soya oil</td>
<td>4</td>
<td>3.87</td>
<td>328</td>
<td>84</td>
<td>73.50</td>
<td>74.3</td>
</tr>
<tr>
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<td>3.77</td>
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<td>73.50</td>
<td>73.4</td>
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<td>84.2</td>
</tr>
</tbody>
</table>
TBP as the carrier that subsists in the organic phase. Based on the results obtained, the MV⁺ is carried across the interface containing D2EHPA and TBP which can be explained as follows.

1. The MV⁺ diffuses from aqueous phase to the surface of two phases interface through aqueous boundary layer.

2. At the aqueous phase interface, the MV⁺ forms a neutral ion pair complex with TBP or D2EHPA.

3. The formed complex of MV⁺ and TBP or D2EHPA then diffuses through the organic phase due to the potential gradient between aqueous and organic phases.

4. Then on the organic phase, the MV⁺ is exchanged with a proton and is released.

5. Finally, the free D2EHPA or TBP acid is diffused back to the aqueous phase and the cycle is repeated (Drapala, 2004).

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**Fig. 1:** Interaction plot for pH and Carriers

**Fig. 2:** Interaction plot for Vegetable oils and Carriers
Methyl violet was extracted from synthetic aqueous solution by liquid-liquid extraction. Crude palm and soy bean oil were used as solvent and D2EHPA and TBP were used as carriers. Based on factorial design of experiments the optimum conditions for dye removal varied from 89% to 82% in 5 rapid mix followed by 60 minutes slow mixing. The aqueous to organic phase volume ratio (A/O) was fixed. pH varied from 1-6 and extraction study was carried out at [TBP]: 200mM and [D2EHPA]: 75mM.

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Abbas F.M. Alkarkhi, is an Associate Professor in Environmental Sciences at the School of Industrial Technology, Universiti Sains Malaysia
Phytochemical Analysis and Cytotoxicity Studies of Curcuma amada rhizomes in BHK-21 Cells

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Abstract. Curcuma amada is medicinal plant which is used in several traditional medicines to cure various diseases. Curcuma amada has been shown to possess anti-inflammatory, antioxidant and antitumor properties. In this investigation, it had been planned to study its anticancer properties in BHK-21 cells. Acetone, Methanol, Ethanol and aqueous extracts of the rhizomes of Curcuma amada were screened for their anticancer properties. The cells were seeded with all the extracts separately and then allowed to grow for 24hrs; the cell growth was inhibited within 24hrs. The cytopathology observed were included rounding and clumping of cells, detachment of cells, flagging of cells and apoptosis. Methanolic and ethanolic extracts showed better response than that of its aqueous and acetone extract. The concentration of 10 mg/ml of ethanolic extract inhibited the cancerous cell growth.

Key words: BHK-21 cells, Curcuma amada, Phytochemical, Cytotoxicity

1. INTRODUCTION

Medicinal plants are a rich source of numerous pharmacologically active molecules. India is a continent with wide field of diversity. This diversity includes both flora as well as fauna. This variation is due to the varied climatic condition, vegetation, topography etc. resulting in enriched heterogeneity. As a result, many such herbs are present with increased medicinal value that is left unnoticed. These herbs may possess medicinal values, domestic values and therapeutic values (Sofowara, 1993). Curcuma amada belonging to Zingiberaceae family is known as mango ginger in English; Manghainchi and Kathumachal in Malayalam and Suraniyika in Sanskrit (Wealth of India, 1952; Warrier et al., 1994; Kirtikar and Basu, 1984). The rhizome, the portion of the plant used medicinally, is usually boiled, cleaned, and dried, yielding a yellow powder. Dried Curcuma amada is the source of the spice turmeric, the ingredient that gives curry powder its characteristic yellow color. Curcuma amada is used extensively in foods for both, its flavor and color, as well as having a long tradition of use in the Chinese and Ayurvedic systems of medicine, particularly as an anti-inflammatory and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic. Curcuma amada can also be applied topically in poultices to relieve pain and inflammation (Govindarajan, 1980).

Various researches had focused on turmeric’s antioxidant, hepatoprotective, anti-inflammatory, anticarcinogenic, and antimicrobial properties, in addition to its use in cardiovascular disease and gastrointestinal disorders (Leung, 1980). The rhizome of the plant Curcuma amada has been used for centuries in traditional medicine and is known to have cancer preventive or therapeutic capabilities (Anand et al., 2008). It has been shown to suppress multiple signaling pathways and inhibit cell proliferation, invasion, metastasis, and angiogenesis (Kunnumakkara et al., 2008; Duvoix et al., 2005). Its safety combined with its low cost, and multiple targeting potential makes Curcuma amada an ideal agent to be explored for prevention and treatment of various cancers and fits very well as a candidate for chemoprevention by edible phytochemicals (Aggarwal, 2008; Gupta, 2007). This study was a step towards evaluation of the plant against cancer. Methanolic, ethanolic, acetone and aqueous extract of the rhizome of Curcuma amada were screened for their anticancer properties. The result of the phytochemical examination of the flowers of this plant is described in this communication.

2. MATERIALS AND METHODS

Plants have been selected from high altitude area (1600m from sea level) from the polyhouse nursery of Institute of Biotechnology, Patwadangar (Nainital), Uttarakhand. Rhizome of Curcuma amada...
were collected and washed with tap water thrice. Washing was again repeated five times by using distilled water. Then the rhizome of *Curcuma amada* were air dried and thereaf ter kept in incubator at 37°C for 24 hrs. The dried material was then crushed in mechanical grinder in order to make fine powder which was stored at room temperature for further use.

3. EXTRACT PREPARATION

3.1. Aqueous Extract

The aqueous extract was prepared according to the standard method with slight modifications. Now, 5g of rhizome powder was mixed in 120 ml of water and was kept in incubator shaker at 36°C and 100 rpm. The extract so obtained was evaporated to drying through heating in a china dish. Dry extract was then scrapped off, weighed and reconstituted in normal saline (Marks et al., 2008).

3.2. Solvent Extraction

Ethanol, Acetone and methanol extracts were prepared in Soxhlet’s apparatus. Soxhlet’s extraction was carried out at room temperature. Dried rhizome powder of *Curcuma amada* weighed accurately 5gm and taken in thimble and subjected to extraction in a Soxhlet’s apparatus at room temperature using ethanol (150ml), acetone (170ml) and methanol (165ml) (Govindachari et al., 1999). The extract obtained was first filtered through Whatman No. 1 filter paper and solvent was then removed under reduced pressure in a vacuumed rotary evaporator and dried. The dried extract was stored in airtight containers for further studies.

For the present study, BHK-21 cells were cultured in 24 well sterile polystyrene plates using GMEM media supplemented with 5% fetal bovine serum as per standard procedure. The cells were seeded into 24 well sterile polystyrene plates and were incubated for 24 hours at 37°C. Thereafter, the medium was removed and 0.5ml of each dilution (10mg,1mg,10μgm) of each extracts added to the assigned wells, Control were also kept (medium without test sample) and triplicate sets of each dilution were maintained. Finally the cells were incubated for 24 hours at 37°C and thereafter, examined under inverted microscope for their morphological studies.

3.3. Confirmatory Tests

3.3.1. MTT Assay

The MTT Assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) into a dark formazan product that is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cell lines. It was performed as, prepared an MTT stock solution of 5 mg ml–1 in phosphate-buffered saline (PBS), pH 7.5, and filter through a 0.22-μ filter to sterilize and the small amount of insoluble residue was removed. Add10 μl of MTT (5mg ml–1), after 24 h of incubation and the cells were further incubated in incubator at 37°C for 3 h. Then 100 μl 0.04 M HCl in propan-2-ol to eachwell were added and mixed thoroughly to dissolve insoluble blue formazan crystals. The Plates were read on a micro-ELISA reader using a test wavelength of 570nm (Mosmann, 1983).

3.3.2. Neutral Red Assay

Neutral red (3-amino-7-dimethyl-2-methylphenazine hydrochloride) is a water soluble, weakly basic, supravital dye that accumulates in lysosomes of viable cells. The neutral red (NR) assay is an invito cell viability test that was developed and extensively studied for in vitro cytotoxicity determination. After incubation of cells with extracts, 0.33% of NR (NR in PBS) was added in each well and incubated for 1 h at 37°C. Dye-containing medium was removed and the well was washed twice with 150μl well warmed PBS. The cells were then lysed with 125 μl of 50% of v/v mixture of ethanol and 0.1Mmonobasic sodium phosphate to solubilise the neutral red. The plate was then incubated for 15 min and take O.D at 550 nm (Flick and Gifford, 1984).

3.3.3. Cytotoxicity % = A-B/A X 100

A = O.D of untreated well;
B = O.D of wells treated with plant extract.

3.4. Phytochemical Analysis

In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents. Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of various infections. These are the qualitative tests performed to analyze the presence or absence of various phytochemicals such as alkaloids, tannins,
flavonoids etc. in plant extract (Wagner and Bladt, 1996).

4. RESULTS

Phytochemical analysis of Curcuma amada methanolic extract, showed positive result for alkaloids, tannins, phenolic compounds, phytosterols, terpenoids, saponins and flavonoids. Aqueous extract of Curcuma amada showed the presence of reducing sugar, Amino acid, steroids, cardiac glycosides, saponins and alkaloids. Ethanolic extract of Curcuma amada showed the presence of reducing sugar, amino acid, steroids, cardiac glycosides, antheraquione glycosides, saponins and alkaloids. Acetone extract of Curcuma amada showed the presence of phenolic compounds, phytosterols, terpenoids, saponins and amino acid.

The cells were observed after 24 hours to record the changes in morphology. The induction of apoptosis resulted in cell shrinkage, eventually leading to the formation of apoptotic bodies. The cells also got detached from the substratum shown by flagging of the cells and also they formed clumps after detachment from the substratum. The morphological changes revealed that the ethanolic and methanolic extract of Curcuma amada rhizome were better than the aqueous and acetone extract of rhizome. The shapes of cells were changed. Elongated cell turn to round cell and clumping was observed. The cytotoxicity study also clearly indicated, that the ethanolic and methanolic extracts of Curcuma amada rhizome killed more cells then that of aqueous and acetone extracts.

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Extracts</th>
<th>10mg/ml</th>
<th>1mg/ml</th>
<th>10μgm/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Aqueous extract</td>
<td>69.84±3.51</td>
<td>67.99±3.63</td>
<td>64.65±3.43</td>
</tr>
<tr>
<td>2.</td>
<td>Acetone extract</td>
<td>41.22±3.18</td>
<td>40.91±3.42</td>
<td>38.47±3.63</td>
</tr>
<tr>
<td>3.</td>
<td>Ethanolic extract</td>
<td>84.32±3.21</td>
<td>82.54±3.09</td>
<td>80.06±3.11</td>
</tr>
<tr>
<td>4.</td>
<td>Methanolic extract</td>
<td>74.29±3.07</td>
<td>71.64±3.57</td>
<td>70.41±3.27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Extracts</th>
<th>10mg/ml</th>
<th>1mg/ml</th>
<th>10μgm/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Aqueous extract</td>
<td>69.97±2.47</td>
<td>68.92±2.51</td>
<td>65.39±2.37</td>
</tr>
<tr>
<td>2.</td>
<td>Acetone extract</td>
<td>43.27±2.11</td>
<td>41.83±2.18</td>
<td>39.32±2.23</td>
</tr>
<tr>
<td>3.</td>
<td>Ethanolic extract</td>
<td>87.63±2.03</td>
<td>85.42±2.81</td>
<td>81.35±2.42</td>
</tr>
<tr>
<td>4.</td>
<td>Methanolic extract</td>
<td>76.31±2.21</td>
<td>74.61±2.33</td>
<td>70.39±2.17</td>
</tr>
</tbody>
</table>

Cytotoxicity measured by MTT Assay of Curcuma amada rhizome of aqueous extract at 10mg/ml was found 69%, at 1mg/ml the cytotoxicity was found 67% and at 10μgm/ml the cytotoxicity was 64%. Whereas, ethanolic extract of Curcuma amada rhizome at 10mg/ml was found 84%, at 1mg/ml the cytotoxicity was found 82% and at 10μgm/ml the cytotoxicity was 80%. Methanolic extract of Curcuma amada rhizome at 10mg/ml was found 74%, at 1mg/ml the cytotoxicity was found 71% and at 10μgm/ml the cytotoxicity was 70%. (Table 1) Acetone extract of Curcuma amada rhizome at 10mg/ml was found 41%, at 1mg/ml the cytotoxicity was found 40% and at 10μgm/ml the cytotoxicity was 38%.

Cytotoxicity measured by NR Assay of Curcuma amada rhizome of aqueous extract at 10mg/ml was found 69%, at 1mg/ml the cytotoxicity was found 68% and at 10μgm/ml the cytotoxicity was 65%. Whereas, ethanolic extract of Curcuma amada rhizome at 10mg/ml was found 87%, at 1mg/ml the cytotoxicity was found 85% and at 10μgm/ml the cytotoxicity was 81% (Table 2). Methanolic extract of Curcuma amada rhizome at 10mg/ml was found 76%, at 1mg/ml the cytotoxicity was found 74% and at 10μgm/ml the cytotoxicity was 70%. Acetone extract of Curcuma amada rhizome at 10mg/ml was found 43%, at 1mg/ml the cytotoxicity was found 41% and at 10μgm/ml the cytotoxicity was 39%.

4. DISCUSSION

Today there is a wide range of medicinal plant parts which include the flowers, leaves, stem, fruits and root extracts which are used as powerful raw drugs possessing a variety of antimicrobial and healing properties. The phytochemical screening of the rhizome of Curcuma amada showed the presence of secondary metabolites including phenols, saponins, tannins and coumarins which had great medicinal properties. In addition, there are several reports to show Curcuma amada species for having potent antimicrobial chemicals (Kannan et al., 2013).

Animal studies involving rats and mice, as well as in vitro studies utilizing human cell lines, have demonstrated Curcuma amada rhizome extract ability to inhibit carcinogenesis at three stages: tumor promotion, 18 angiogenesis, Thaloor et al., 1998 and tumor growth, Limtrakul et al., 1997). In two studies of colon and prostate cancer, Curcuma amada...
rhizome extract inhibited cell proliferation and tumor growth (Hanif et al., 1997; Dorai et al., 2001). Curcuma amada rhizome extract and curcumin are also capable of suppressing the activity of several common mutagens and carcinogens in a variety of cell types in both in vitro and in vivo studies (Mehta and Moon, 1991; Soudamini and Kuttan, 1989; Azuine and Bhide, 1992; Boone et al., 1992).

![Rhizome of Curcuma amada](image1)

![Normal BHK-21 cells](image2)

![Cells treated with extract of rhizome of Curcuma amada](image3)

The anticarcinogenic effects of *Curcuma amada* rhizome extract and curcumin are due to direct antioxidant and free-radical scavenging effects, as well as their ability to indirectly increase glutathione levels, thereby aiding in hepatic detoxification of mutagens and carcinogens, and inhibiting nitrosamine formation. Numerous animal, *in vitro*, and *in vivo* studies have demonstrated the anti-carcinogenic effects of *Curcuma amada* rhizome extract and its flavonoid component curcumin against colon (Chauhan, 2002; Reddy and Rao, 2002), breast, (Ramachandran et al., 2002; Somasundaram et al., 2002; Shao et al., 2002) and prostate cancers, as well as melanoma (Hour et al., 2002). A human study of 25 individuals at high risk of neoplasia or with pre-malignant lesions noted histologic improvement in one of two patients with recently resected bladder cancer, two of seven patients with oral leukoplakia, one of six patients with intestinal metaplasia of the stomach, one of four patients with cervical intraepithelial neoplasm, and two of six patients with Bowen’s disease. More clinical trials need to be performed to further elucidate the potential of this botanical in cancer prevention and treatment (Bush et al., 2001; Cheng et al., 2001). Curcuma zedoaria and Curcuma amada rhizome solvent extracts were evaluated for their anticancer and antioxidant activity. The isopropyl extract of *C. zedoaria* exhibited high anticancer activity compared to acetone extract of *C. amada*. Crude protein of *C. zedoaria* showed good anticancer activity when compared to crude protein of *C. amada*. Acetone extract of *C. zedoaria* showed high radical scavenging activity of 88.7% and superoxide scavenging activity recording 83.15%. Acetone extract of *C. zedoaria* showed 82.5% hydroxyl radical scavenging activity (Kumar et al., 2012). Muthu kumar et al. (2012) had reported the anticancer and antioxidant activity of *Curcuma zedoaria* and *Curcuma amada* rhizome extracts. The isopropyl extract of *C. zedoaria* exhibited high anticancer activity compared to acetone extract of *C. amada*. Crude protein of *C. zedoaria* showed good anticancer activity when compared to crude protein of *C. amada*. Acetone extract of *C. zedoaria* showed high radical scavenging activity of 88.7% and superoxide scavenging activity recording 83.15%. Acetone extract of *C. zedoaria* showed 82.5% hydroxyl radical scavenging activity.

Thus, in the present investigation, on the basis of observed encouraging cytotoxic effects by the *in-vitro* bioassays, it can be revealed that methanolic and ethanolic extracts of *Curcuma amada* rhizome extracts had promising anticancer bio efficacy than its ethanolic extract and must have some phytochemical
moiety in the leaves of this plant which might be responsible for observed beneficial effects. It is suggested that the detailed in-vivo studies should be carried out in animal experimental model of cancer to further prove the anticancerous activity of Curcuma amada rhizome extracts. Moreover, the inhibitory effect of all the solvent extract and aqueous extract of Curcuma amada rhizome were maximal at 24 hours. These observations suggest that active principle might get metabolized or get inactivated during culture process and is no more available to impose growth inhibitory effect. However, these assumptions need further investigation. In contrast to Curcuma amada rhizome aqueous and acetone extract showed a minor growth promoting activity in dose range 10gm/ml -10 μg/ml. These results, suggest accumulation of cell growth promoter sin ethanolic extract that may have coexisted in the plant rhizome along with cytotoxic activity. However, there is no report which specifically describes any tumor growth promoting activity associated with this plant.

5. CONCLUSION

The active phytochemical constituents present in Curcuma amada rhizome extract imparts high therapeutic properties that can prevent, various infection, flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic and other ailments. The aqueous, methanolic, acetone and ethanolic extracts were tested on BHK-21 cells, which showed cytotoxic effects on the cancer cells. Assessing its anticancer activity in our study indicates for the first time that Curcuma amada rhizome extract act as an cytotoxic inducing property against BHK-21 cells. It therefore provides an important lead for development of anti-cancer therapeutics for management of cancer.

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Full Length Research Paper

Upland Rice Root Parameters and Their Relationship on Utilizing Different Levels of Applied Zinc

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Abstract. Global efforts are under way to improve the Zn concentrations in rice to increase Zn in diets. Zinc uptake in relation to morphological root parameters among 7 upland rice varieties was studied by conducting a solution culture experiment using modified Yoshida solution in Agriculture Faculty of University Putra Malaysia. Five zinc levels were developed by the addition of 0, 5, 10, 20, 30 mg L⁻¹ ZnSO₄. Seedlings were harvested in week 4. Zn uptake in roots of upland rice showed significant differences among all varieties. Zn uptake significantly increased at all rates. Other root parameters (length, average diameter, surface area, volume, and number of root tips) did not show any significant differences in 0 to 20 mg Zn L⁻¹, but they decreased significantly in 30 mg Zn L⁻¹ in 4th week of observation.

Key words: Zinc, Rice, Root Morphology, Zn Uptake

1. INTRODUCTION

Zinc is essential for the normal healthy growth and reproduction of plants. The normal concentration of this element is 25 to 150 mg kg⁻¹ in plants. Deficiencies of Zn are usually associated with concentrations of less than 20 mg kg⁻¹, and toxicities will occur when the Zn leaf concentration exceeds 400 mg L⁻¹ (Tisdale et al., 1993). Cultivars differ in their ability to take up Zn, which may be caused by differences in zinc translocation and utilization, differential accumulation of nutrients that interact with Zn and differences in plant roots to exploit for soil Zn (Tisdale et al., 1993).

Rice, the main staple food of Asia, is inherently very low in Zn and its high consumption relative to other foods contributes to high incidence of Zn deficiency in human populations in Asia (Gibson et al., 2007; Stein et al., 2007). Rice yield and growth are very sensitive to Zn and it’s an important plant nutrient for plant vigor after N and P. Upland rice is water saving rice production system depending on irrigation water management and anticipated yield (Wang et al., 2009). Upland rice needs to have a deeper rooting and a higher root length and density than lowland rice cultivars because of the limited water availability under aerobic as compared to flooded conditions (Matsuo et al., 2010). The primary source of Zn for rice plants is through root uptake (Welch and Graham, 2002). To increase Zn uptake by roots, the Zn availability in the rhizosphere must be increased (Welch and Shuman, 1995).

Some researchers reported that under nutrient-deficient conditions, plants tend to alter their root size and morphology for efficient nutrient acquisition. Enhanced root growth under Zn deficiency, both in length and number of roots, has been associated with Zn-deficiency tolerance of lowland rice genotypes. In addition researchers showed moderate Zn deficiency enhanced both root length and the number of total root tips to a greater extent in a Zn-deficiency-tolerant genotype than in a susceptible one, but severe Zn deficiency reduced root growth in both the genotypes compared with Zn-sufficient conditions (Chen et al., 2009). Most recent studies in rice suggest that among numerous other mechanisms, Zn uptake is most important. However, there has been a little critical appraisal of root traits. This study was undertaken to evaluate the effect of different rates of Zinc on root morphological traits among different upland rice genotypes in Malaysia.

2. MATERIALS AND METHODS

Seeds of seven upland varieties (Table 1) were collected from different parts of Malaysia. This study was conducted at Faculty of Agriculture, University Putra Malaysia from April to May 2012. Five treatments of different Zinc concentrations (0, 5, 10, 20 and 30 mg L⁻¹) were applied on the seven upland rice varieties (ZnSO₄·7H₂O as source of Zn was...
applied). Experimental units were grown in Yoshida solution culture (Yoshida, 1981) in growth chamber and pH was daily adjusted on 5.5. Plants were irrigated twice a day and seedlings harvested in 4th week. The roots that developed after 28 days were scanned using WinRHIZO root scanning software.

Root parameters, such as volume, surface area, average diameter, length and number of root tips were recorded. Dry-matter weights and root Zn content were determined. This study was repeated three times and conducted by RCBD. The data obtained were subjected to ANOVA using the SAS 9.2 version.

Table 1: Sign and name of varieties

<table>
<thead>
<tr>
<th>No.</th>
<th>Sign</th>
<th>Name of Var.</th>
<th>State of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V₁</td>
<td>Bertih</td>
<td>Pahang</td>
</tr>
<tr>
<td>2</td>
<td>V₂</td>
<td>Tenom</td>
<td>Sabah</td>
</tr>
<tr>
<td>3</td>
<td>V₃</td>
<td>Kesum</td>
<td>Pahang</td>
</tr>
<tr>
<td>4</td>
<td>V₄</td>
<td>Sintuk</td>
<td>Pahang</td>
</tr>
<tr>
<td>5</td>
<td>V₅</td>
<td>Polut wangi</td>
<td>Pahang</td>
</tr>
<tr>
<td>6</td>
<td>V₆</td>
<td>Hita</td>
<td>Pahang</td>
</tr>
<tr>
<td>7</td>
<td>V₇</td>
<td>Nabawan</td>
<td>Sabah</td>
</tr>
</tbody>
</table>

3. RESULTS AND DISCUSSIONS

Seeds of seven upland varieties (Table 1) were collected from different parts of Malaysia. This study was conducted at Faculty of Agriculture, University Putra Malaysia from April to May 2012. Five treatments of different Zinc concentrations (0, 5, 10, 20 and 30 mg L⁻¹) were applied on the seven upland rice varieties (ZnSO₄·7H₂O as source of Zn was applied). Experimental units were grown in Yoshida solution culture (Yoshida, 1981) in growth chamber and pH was daily adjusted on 5.5. Plants were irrigated twice a day and seedlings harvested in 4th week. The roots that developed after 28 days were scanned using WinRHIZO root scanning software.

High level of Zinc showed severe phytotoxic effects rice and significantly inhibited its growth by interfering with certain important metabolic process were also observed by (Alam et al., 2002) and (Ebbs and Kochian, 1998). As clearly seen in the Table 4, Table 5 and Table 6, the effects of application of different rates of zinc on root surface area, number of root tips, and volume, followed the same trend as root
length. The effects of application of different rates of zinc on average root diameter showed that this root parameter didn’t have any significant differences between all rates in most of the varieties (Table 7).

### Table 4: Root surface area of varieties at different rate of zinc

<table>
<thead>
<tr>
<th>Variety</th>
<th>0</th>
<th>5(mgL⁻¹)</th>
<th>10(mgL⁻¹)</th>
<th>20(mgL⁻¹)</th>
<th>30(mgL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>25.127 c</td>
<td>31.117 ab</td>
<td>32.000 ab</td>
<td>34.900 a</td>
<td>28.347 bc</td>
</tr>
<tr>
<td>V2</td>
<td>30.643 b</td>
<td>47.640 a</td>
<td>31.133 b</td>
<td>34.570 b</td>
<td>34.183 b</td>
</tr>
<tr>
<td>V3</td>
<td>26.970 c</td>
<td>34.950 b</td>
<td>33.453 b</td>
<td>40.380 a</td>
<td>31.327 bc</td>
</tr>
<tr>
<td>V4</td>
<td>31.333 b</td>
<td>32.473 b</td>
<td>37.910 a</td>
<td>38.577 a</td>
<td>31.737 b</td>
</tr>
<tr>
<td>V5</td>
<td>36.243 c</td>
<td>46.783 a</td>
<td>41.963 b</td>
<td>40.327 bc</td>
<td>35.153 c</td>
</tr>
<tr>
<td>V6</td>
<td>34.960 b</td>
<td>32.073 b</td>
<td>44.450 a</td>
<td>41.900 a</td>
<td>40.193 a</td>
</tr>
<tr>
<td>V7</td>
<td>30.717 c</td>
<td>34.433 b</td>
<td>41.560 a</td>
<td>44.733 a</td>
<td>35.133 b</td>
</tr>
</tbody>
</table>

@Means in a column with the same letters are not significantly different at 5% level

### Table 5: Number of root tips of varieties at different rate of zinc

<table>
<thead>
<tr>
<th>Variety</th>
<th>0</th>
<th>5(mgL⁻¹)</th>
<th>10(mgL⁻¹)</th>
<th>20(mgL⁻¹)</th>
<th>30(mgL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>328.67 d</td>
<td>487.33 b</td>
<td>580.67 a</td>
<td>580.00 a</td>
<td>354.00 c</td>
</tr>
<tr>
<td>V2</td>
<td>505.67 c</td>
<td>867.00 a</td>
<td>596.67 b</td>
<td>751.33 a</td>
<td>627.67 b</td>
</tr>
<tr>
<td>V3</td>
<td>472.67 c</td>
<td>627.00 b</td>
<td>674.67 b</td>
<td>751.33 a</td>
<td>627.67 b</td>
</tr>
<tr>
<td>V4</td>
<td>560.67 b</td>
<td>607.67 ab</td>
<td>629.33 ab</td>
<td>751.00 a</td>
<td>574.67 b</td>
</tr>
<tr>
<td>V5</td>
<td>570.00 b</td>
<td>680.00 ab</td>
<td>740.67 ab</td>
<td>813.67 a</td>
<td>648.00 ab</td>
</tr>
<tr>
<td>V6</td>
<td>674.67 c</td>
<td>548.67 b</td>
<td>738.67 b</td>
<td>825.00 a</td>
<td>730.67 b</td>
</tr>
<tr>
<td>V7</td>
<td>547.33 c</td>
<td>816.33 a</td>
<td>670.67 b</td>
<td>640.67 b</td>
<td>624.33 b</td>
</tr>
</tbody>
</table>

@Means in a column with the same letters are not significantly different at 5% level

### Table 6: Root volume of upland rice varieties at different rate of zinc

<table>
<thead>
<tr>
<th>Variety</th>
<th>0</th>
<th>5(mgL⁻¹)</th>
<th>10(mgL⁻¹)</th>
<th>20(mgL⁻¹)</th>
<th>30(mgL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>0.363 b</td>
<td>0.377 b</td>
<td>0.427 b</td>
<td>0.483 a</td>
<td>0.363 b</td>
</tr>
<tr>
<td>V2</td>
<td>0.363 b</td>
<td>0.420 b</td>
<td>0.427 b</td>
<td>0.503 a</td>
<td>0.420 b</td>
</tr>
<tr>
<td>V3</td>
<td>0.337 b</td>
<td>0.457 a</td>
<td>0.500 a</td>
<td>0.517 a</td>
<td>0.497 a</td>
</tr>
<tr>
<td>V4</td>
<td>0.273 c</td>
<td>0.383 b</td>
<td>0.450 ab</td>
<td>0.510 a</td>
<td>0.383 a</td>
</tr>
<tr>
<td>V5</td>
<td>0.467 a</td>
<td>0.577 a</td>
<td>0.523 a</td>
<td>0.517 a</td>
<td>0.467 a</td>
</tr>
<tr>
<td>V6</td>
<td>0.417 c</td>
<td>0.450 bc</td>
<td>0.597 a</td>
<td>0.523 ab</td>
<td>0.517 ab</td>
</tr>
<tr>
<td>V7</td>
<td>0.417 b</td>
<td>0.563 a</td>
<td>0.523 a</td>
<td>0.590 a</td>
<td>0.413 b</td>
</tr>
</tbody>
</table>

@Means in a column with the same letters are not significantly different at 5% level

### Table 7: Average root diameter of rice varieties at different rate of zinc

<table>
<thead>
<tr>
<th>Variety</th>
<th>0</th>
<th>5(mgL⁻¹)</th>
<th>10(mgL⁻¹)</th>
<th>20(mgL⁻¹)</th>
<th>30(mgL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>0.55 b</td>
<td>0.56 a</td>
<td>0.57 a</td>
<td>0.58 a</td>
<td>0.56 a</td>
</tr>
<tr>
<td>V2</td>
<td>0.55 a</td>
<td>0.55 a</td>
<td>0.55 a</td>
<td>0.57 a</td>
<td>0.55 a</td>
</tr>
<tr>
<td>V3</td>
<td>0.53 b</td>
<td>0.55 b</td>
<td>0.55 b</td>
<td>0.57 a</td>
<td>0.54 b</td>
</tr>
<tr>
<td>V4</td>
<td>0.54 a</td>
<td>0.54 a</td>
<td>0.54 a</td>
<td>0.55 a</td>
<td>0.55 a</td>
</tr>
<tr>
<td>V5</td>
<td>0.53 c</td>
<td>0.55 ab</td>
<td>0.55 ab</td>
<td>0.57 a</td>
<td>0.54 bc</td>
</tr>
<tr>
<td>V6</td>
<td>0.53 a</td>
<td>0.54 a</td>
<td>0.55 a</td>
<td>0.55 a</td>
<td>0.54 a</td>
</tr>
<tr>
<td>V7</td>
<td>0.53 a</td>
<td>0.55 a</td>
<td>0.56 a</td>
<td>0.57 a</td>
<td>0.55 a</td>
</tr>
</tbody>
</table>

@Means in a column with the same letters are not significantly different at 5% level
4. CONCLUSION

Increase of Zinc rate has a positive effect on morphological root parameters up to 20 mgL$^{-1}$. Soluble forms of Zn are readily available to plants and the uptake of Zn has been reported to be linear with concentration in the nutrient solution and soils. In addition, plants grown in Zn-contaminated soils accumulate a great proportion of the metal in the roots (Kabata-Pendias, 2000). This shows that Zn in high concentration causes root growth disorder. Results of this study showed at 30 mg ZnL$^{-1}$ young plants died, possibly due to toxic effect of Zn. Sensitive plant species are reported to be retarded in growth when their tissues contain 20 to 200 mg Zn kg$^{-1}$. However, the upper toxic levels range in various plants are from 100 to 500 mg kg$^{-1}$ (DW) (Kabata-Pendias, 2000).

REFERENCES


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