ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 8, pp. 12941-12946, August, 2016

International Journal of Recent Scientific Research

Research Article

IMPROVEMENT OF COBALT CHLORIDE CAUSED STRESS IN TERMS OF MORPHOMETRIC, PIGMENTAL, BIOCHEMICAL AND ENZYMATIC CHARACTERISTICS OF *VIGNA UNGUICULATA* (L.) BY BIOSORPTION USING *DICTYOTA DICHOTOMA*

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ARTICLE INFO

Article History:

Received 05th May, 2016
Received in revised form 21st June, 2016
Accepted 06th July, 2016
Published online 28th August, 2016

Key Words:

Biosorption, Cowpea, *Dictyota*, Cobalt toxicity, Antioxidants and Atomic Absorption Spectroscopy

ABSTRACT

Pollution to the environment by heavy metals is mainly due to human activities. The presence of heavy metals even in traces is toxic to both plants and animals. The excess of cobalt can affect the plant's physiological functions. In order to control cobalt heavy metal soil pollution by a low cost technique an attempt was made employing biosorption using *Dictyota dichotoma*. In this study, the seedlings of (cowpea) *Vigna unguiculata* (L) were treated with various concentrations of cobalt chloride and its impact on the morphometric, biochemical and enzymatic characteristics were studied. Eight days after treatment with different concentrations of cobalt chloride (2mM, 4mM, 6mM, 8mM, & 10 mM), the growth parameters such as leaf area, fresh weight, dry weight, shoot length, root length were found decreased than in the control. Biochemical parameters such as soluble sugar and protein content were decreased with the increase in the concentrations of cobalt chloride. On the contrary the contents of free amino acid, proline and leaf nitrate were increased with increase in the concentrations of cobalt chloride. The activities of enzymes such as catalase and peroxidase barring nitrate reductase were found increased with the increase in the concentration of cobalt chloride. Application of 6mM cobalt chloride solution treated with various concentrations of *Dictyota dichotoma* such as 2gm/L, 4gm/L & 6gm/L & 8gm/L on the experimental plants has brought about changes in the suppressed characteristics showing relief from stress due to cobalt chloride. Atomic Absorption Spectroscopy (AAS) technique was employed to confirm the presence of cobalt in the treated and control plants. Comparison of the values of treated plants with control reveals that cobalt chloride has seriously affected the (cowpea) *Vigna unguiculata* (L) plants and *Dictyota dichotoma* is effectively biosorbed the cobalt heavy metal.

INTRODUCTION

Pollution is one of the major threats to environment, particularly soil and water pollution. The pollution occurs by various types of pollutants, heavy metals are the important chemical pollutants. Heavy metals are included in the main category of environmental pollutants as they can remain in the environment for long periods; their accumulation is potentially hazardous to humans, animals and plants (Webber 1981; Baker *et.al*. 1994; Deram *et.al*. 2000; Jayakumar *et.al*. 2008; Abdul Jaleel *et.al*. 2009). The heavy metal pollution of surface soils due to intense industrialization and urbanization has become a serious concern in many developing countries (Mireles *et.al*. 2012; Wei and Yang 2010; Yaylalı-Abanuz 2011). Many studies reported that Co at toxic levels inhibit pollen germination, pollen tube growth Levent Tuna *et.al*. (2002) & inhibit seed germination, causing ultra – structural changes & may cause inhibition in growth of plumule & radicals Ayaz and Kadioglu (1997). The cobalt is one of the heavy metal cause soil pollution in excess to destroy crop plants life. Cobalt stress on growth and biochemical constituents of cowpea (*Vigna unguiculata* (L.) Walp.) With specific emphasize on antioxidant enzymes activities which are the defence mechanism to any type of abiotic stress Vijayarengan (2012). Biosorption is a term that describes the removal of heavy metal, by the passive binding to non-living biomass from an aqueous solutions. Biosorption uses inexpensive dry biomass to extract industrial effluents of toxic heavy metals. The biosorption is a process in which solids of natural origin are employed for binding heavy metals .The biomass can be composed of algae, mosses, fungi, bacteria, and various plant species. It is a promising alternative method to treat industrial effluents, mainly because of its low cost and high metal

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binding capacity. The alga can be collected and/or cultivated in many parts of the world, factor that has encouraged the development of new biosorbent materials using biomass. Cost effective technologies or sorbents for treatment of metal contaminated waste streams are needed Bailey *et.al.* (1999). A number of workers investigated their feasibility of using cheaply available marine or fresh water algae for heavy metal removal (Darnall *et.al.* 1986; Holan *et.al.* 1993). Seaweeds are readily available in large quantities for the development of highly effective biosorbent materials. It was also found that the biosorption capacities were significantly affected by solution pH, with higher pH favoring higher metal-ion removal Sheng *et.al.* (2004). The passive removal of toxic heavy metals by brown marine algae via biosorption was reported by Davis *et al.* (2003a & b), who attributed this property to cell wall polysaccharides like alginate and fucoidan. Biosorption properties of a few algae are accredited to their cell-wall polysaccharides like alginate and fucoidan, which have a high affinity for divalent cations (Fourest *et.al.* 1994; Puranik *et.al.* 1999; Khoo and Ting, 2001; Chen *et.al.* 2002; Davis *et.al.* 2003b).

MATERIALS AND METHODS

For both control and experimental plants seeds were allowed to grow in uniform mixed red, black and sandy soil in 1:1:1 ratio and allowed to germinate. After ten days, the seedling of *Vigna unguiculata* (L) were treated with various concentrations of cobalt chloride such as the seaweed *Dictyota dichotoma* was collected from uvari coastal area Tamil nadu, shade dried and finally powdered by milling. (set I).

The 6 mM of cobalt (the concentration at which the toxicity found to be optimum) level based on LSD analysis Zar (1984) was mixed with various amount of *Dictyota dichotoma* seaweed dry powder (2g/L, 4 g/L, 6 g/L & 8 g/L), constantly shaken for 12 hours, filtered and the plants of another set was treated with the filtrate. (set II).

Ten days later plants of both sets, the morphometric characters such as shoot length and root length are measured by scale and expressed in cm^2. The leaf area was measured by conventional graphical method by drawing the outline of the leaf in a graph sheet and counting the small and big squares with in it and represented in cm^2. The fresh weight and dry weight of the seedlings were obtained using an electronic balance.

To quantify the total chlorophyll from leaves, fresh leaves were deveined and cut into small bits. From the pool leaf bits, a sample of 100 mg was weighed. The leaf bits were homogenized in 100% acetone using morter and pestle. The homogenate was centrifuged at 4000 rpm for 5 minutes at room temperature. Extraction with 100% acetone was repeatecd till the pellet becomes pale yellow or white in colour. The supernatant was used for the estimation of photosynthetic pigments. The absorbance was measured at 662 nm, 645 nm and 470 nm for chlorophyll a, chlorophyll b and carotenoids respectively using a systronics spectrophotometer model no: 106. The amount of chlorophyll a, chlorophyll b and total chlorophyll was calculated using the formulae of Welburn and Lichtenthaler (1984).

Chlorophyll a (mg/L = 11.75 x A_{662}-2.35xA_{645}

Chlorophyll b (mg/L = 18.61 x A_{645}-3.96xA_{662}

Total chlorophyll=chlorophyll a+chlorophyll b

The carotenoid content was calculated using the following formula:

$$\text{Xanthophyll + Carotenes} = \frac{1000 \times A470 - 2.27\, C_a - 8.14\, C_b}{227}$$

C_a = Total chlorophyll a
C_b = Total chlorophyll b

The anthocyanin was estimated by inoculating two hundred mg of leaf samples inoculating in 200 ml of extraction medium that consists of methanol, diluted water and HCl in the ratio of 50:20:1 respectively. The incubation was extended to 48 hours at 4ºC in dark condition with agitation. After 48 hours, the solution was collected and the optical density was measured at 630 nm and 657nm. The absorbance value of anthocyanin content was estimated by Mancinelli *et.al.* (1973) method.

Absorbance of anthocyanin = $A_{530} - 0.3 \times A_{657}$

The total soluble sugar was measured by anthrone method likewise the amino acid content was measured by ninhydrin method Jayaraman (1981) The protein content was measured, folin phenol with alkaline cobalt mixture Lowry *et.al.* (1951). The proline was estimated using sulphosalicylic acid, glacial acetic acid Bates *et.al.* (1973). The biochemicals were estimated using standard value.

The *In vivo* nitrate reductase (NR) activity was assayed according to Jaworski *et.al.* (1871). Method with modification. Fresh leaf material (100mg) was incubated in scintillation vials containing 5 mL of incubation medium composed of, 100mM KH_2PO_4-KOH buffer pH 7.5, 200mM KNO3, 1% (v/v) n-propanol and 0.1% (v/v) Triton X 100. Incubation was carried out in dark for one hour at room temperature with occasional shaking. Aliquots of 0.5 ml was taken from the vials and analysed for nitrite after 1 hour incubation. To 0.5 ml distilled water, 1 of 3% sulphanilamide and 1 ml of N-1-N (Naphthyl ethylene-diamine dihydrochloride) were added in quick succession. Fifteen minutes were allowed for colour formation and absorbance was measured at 540 nm. The nitrite was estimated with the help of a standard nitrite curve. The activities of peroxidise and catalase enzyme extracts of the leaves of experimental plants were extracted by grinding per gram of leaf in 5 mL of 100 mM phosphate buffer (pH 6.0) and filtered through a three layered cheese cloth and sun at 3000 rpm for 30 minutes. The supernatant obtained was served as the source for crude enzymes such as peroxidise and catalase.

To assay peroxidise activity, the enzyme extract was added to pyrogallol which gets oxidized to a coloured derivative in the presence of hydrogen peroxide (1% v/v). The amount of pupurogallin formed during the reaction was assayed spectroscopically (Addy and Goodman 1972). To 2 mL of pyrogallol phosphate buffer (0.058 M pyrogallol dissolved in 0.1 M phosphate buffer pH 6.0), an aliqot of 0.1 mL of enzyme extract was added. Then, absorbance was set to zero at 420 nm. To this, 0.5 mL of H_2O_2 (1% v/v) was added. Then, the content was thoroughly mixed and the absorbasnce was measured using systronics model no. 106 spectrophotometer. The difference in

the absorbance at an interval of 20 seconds for a period of 3 minutes was measured. The peroxidise activity was expressed as moles of H_2O_2 reduced per unit enzyme per unit time.

To assay the catalase activity, 3 mL of phosphate buffer was added to 1 mL of H_2O_2 and 1 ml of enzyme extract (Kar and Mishra 1976). The reaction mixture was incubated at 25ºC for 1 minute. The reaction was terminated by the addition of 1 mL of H_2SO_4. The end point was the persistence of pink colour at least for 15 seconds. The catalase activity was expressed in micromoles H_2O_2 catalysed per unit time per mg protein. The heavy metal accumulation of the experimental plants was analysed at the end of the plant life. Heavy metal concentrations in plants were analysed using Baker *et.al.* (1994) method. The plant sample as a whole was washed, dried in oven at 160ºC for 40 minutes and digested in a mixture of nitric acid and perchloric acid (10:1). Then the solution was certifuged at 5000 rpm for 5 minutes and double filtered with Whatmann filter paper no.4 and the filtrate was analysed for cobalt concentration by Atomic Absorption Spectrometry (Shimadzu Model AA – 6300), available in the Science Instrumentation Centre of Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, Tamilnadu.

Statistical Analysis

For the morphometric characters the average value of ten independent samples and for the biochemical readings and enzymatic characters the average value of five were considered. The data were reported as mean ± SE and the percent activity was represented in the parentheses. Statistical analysis (One way ANOVA – Turkey test) was done using the statistical package, Origin – version 7.0.

RESULTS

The results obtained on the effects of different concentration of cobalt chloride were summarized and discussed. The results showed that the morphometric characters such as root length, shoot length, leaf area, fresh weight and dry weight increased in plants supplied with biosorbent treated cobalt chloride with *Ulva lactuca* (Table 1). The levels of chlorophylls and carotenoids increased but the level of anthocyanin decreased (Table 2). Similarly the total soluble sugar and protein increased and the leaf nitrate, total phenol, free amino acid and proline decreased (Table 3). The activity of nitrate reductase activity increased, but the peroxidise and catalase decreased (Table 4). The cobalt content of the treated and control seedling of *Vigna unguiculata* (L) was finally estimated using Atomic absorption spectroscopy study (Table 5).

DISCUSSION

Abiotic stresses like heavy metal stress, air pollutants stress etc., negatively affect processes associated with biomass production and seed yield in almost all major field grown crops Purves (1985) Heavy metals caused significant decreases in growth and protein content. Cd was the most toxic metal followed by Co, Hg, Mn, Pb, and Cr and these toxic HM effects on the plant growth, nitrogen content in different plant parts, and protein content in seeds Abdul Ghani (2010). A large range of biomass, principally bacteria, algae, seagrasses, crab shells, yeasts, and fungi have received increasing attention for heavy metal ion removal and recovery.

Table 1 Effects of raw and biosorbent treated cobalt chloride on the morphometric characters of *Vigna unguiculata* (L).

Concentration	Shoot length	Root length	Leaf area	Fresh weight	Dry weight
Control (H2O)	19.60 ± 0.115 (100)	11.30 ± 0.057 (100)	05.37 ± 0.139 (100)	01.74 ± 0.051 (100)	00.12 ± 0.005 (100)
6 Mm Co	14.60 ± 0.152 (74.49)	08.74 ± 0.024 (77.37)	04.12 ± 0.035 (76.74)	01.04 ± 0.029 (60.15)	00.08 ± 0.003 (69.44)
2g *Dictyota*+ 6mM Co	15.30 ± 0.057 a (78.06)	08.88 ± 0.026 a (78.61)	04.06 ± 0.038 a (75.62)	01.07 ± 0.130 a (61.49)	00.08 ± 0.005 a (66.67)
4g *Dictyota*+ 6mM Co	16.70 ± 0.145 a (84.01)	09.99 ± 0.020 a (88.47)	04.36 ± 0.088 a (81.27)	01.27 ± 0.057 a (73.37)	00.09 ± 0.002 a (78.33)
6g *Dictyota*+ 6mM Co	17.60 ± 0.145 a (88.44)	11.18 ± 0.058 a (98.97)	04.83 ± 0.033 a (89.95)	01.50 ± 0.073 a (86.21)	00.10 ± 0.006 a (91.39)
8g *Dictyota*+ 6mM Co	19.40 ± 0.208 a (101)	11.33 ± 0.033 a (100.3)	05.44 ± 0.159 a (101.4)	01.76 ± 0.057 a (101.5)	00.12 ± 0.003 a (102.8)

Values are an average of six observations. Values in parenthesis are percentage activity with respect to control. Mean ± SE a – 2g, 4g, 6g and 8g *Dictyota dichotoma* treatment compared with control, Significance ($P \leq 0.05$ – Turkey test).

Table 2 Effects of raw and biosorbent treated cobalt chloride on the pigmental characters of *Vigna unguiculata* (L).

Concentration	Chlorophyll a (mg/gLFW)	Chlorophyll b (mg/gLFW)	Total chlorophyll (mg/gLFW)	Carotenoids (mg/gLFW)	Anthocyanin (mg/gLFW)
Control (H2O)	03.52 ± 0.080 (100)	02.01 ± 0.057 (100)	05.54 ± 0.126 (100)	00.64 ± 0.030 (100)	05.79 ± 0.061 (100)
6 Mm Co	01.33 ± 0.101 (37.83)	00.24 ± 0.015 (12.07)	01.57 ± 0.096 (28.48)	00.37 ± 0.023 (51.48)	08.96 ± 0.172 (154.8)
2g *Dictyota*+ 6mM Co	01.42 ± 0.018 a (40.50)	00.33 ± 0.094 a (16.44)	01.75 ± 0.105 a (31.76)	00.45 ± 0.007 a (62.76)	08.81 ± 0.212 a (152.2)
4g *Dictyota*+ 6mM Co	01.96 ± 0.032 a (55.73)	00.94 ± 0.192 a (47.21)	02.91 ± 0.167 a (52.63)	00.58 ± 0.024 a (79.60)	07.80 ± 0.152 a (134.7)
6g *Dictyota*+ 6mM Co	02.78 ± 0.102 a (78.96)	01.7 7± 0.019 a (88.10)	04.55 ± 0.816 a (82.27)	00.69 ± 0.026 a (91.36)	06.84 ± 0.061 a (118.2)
8g *Dictyota*+ 6mM Co	03.58 ± 0.038 a (101.7)	02.06 ± 0.016 a (102.8)	05.72 ± 0.041 a (103.3)	00.71 ± 0.007a (102.4)	06.18 ± 0.117 a (106.8)

Values are an average of six observations. Values in parenthesis are percentage activity with respect to control. Mean ± SE a – 2g, 4g, 6g and 8g *Ulva lactuca* treatment compared with control, Significance ($P \leq 0.05$ – Turkey test).

Table 3 Effects of raw and biosorbent treated cobalt chloride on the biochemical characters of *Vigna unguiculata* (L).

Concentration	Total soluble sugar (mg/gLFW)	Total soluble protein (mg/gLFW)	Amino acid (µMole/g LFW)	Total phenol (µMole/g LFW)	Proline (µMole/g LFW)	Leaf nitrate (µMole/g LFW)
Control (H2O)	06.78 ± 0.044 (100)	19.87 ± 0.373 (100)	14.17 ± 0.075 (100)	03.16 ± 0.035 (100)	05.76 ± 0.111 (100)	00.56 ± 0.007 (100)
6 Mm Co	04.48 ± 0.090 (66.09)	07.37 ± 0.063 (37.08)	26.77 ± 0.200 (188.9)	05.26 ± 0.030 (166.7)	09.01 ± 0.223 (156.4)	00.32 ± 0.004 (57.61)
2g *Dictyota*+ 6mM Co	04.69 ± 0.046 a (69.24)	09.16 ± 0.046 a (46.10)	25.94 ± 0.315 a (183)	04.64 ± 0.258 a (147.1)	07.49 ± 0.043 a (130.1)	00.35 ± 0.002 a (64.11)
4g *Dictyota*+ 6mM Co	04.96 ± 0.025 a (73.12)	12.59 ± 0.709 a (63.38)	23.01 ± 0.989 a (162.3)	04.09 ± 0.025 a (129.7)	06.66 ± 0.035 a (115.7)	00.42 ± 0.001 a (76.24)
6g *Dictyota*+ 6mM Co	05.61 ± 0.120 a (82.75)	15.02 ± 0.988 a (75.62)	17.50 ± 0.687 a (123.5)	03.43 ± 0.037 a (108.6)	06.06 ± 0.089 a (105.4)	00.49 ± 0.0001 a (88.35)
8g *Dictyota*+ 6mM Co	06.72 ± 0.056 a (99.16)	20.08 ± 0.156 a (101.1)	14.21 ± 0.157 a (100.3)	03.18 ± 0.024 a (100.8)	05.81 ± 0.071a (101)	00.56 ± 0.004 a (101.5)

Values are an average of six observations. Values in parenthesis are percentage activity with respect to control. Mean ± SE a – 2g, 4g, 6g and 8g *Ulva lactuca* treatment compared with control, Significance (P ≤ 0.05 – Turkey test).

Table 4 Effects of raw and biosorbent treated cobalt chloride on the enzymatic characters of *Vigna unguiculata* (L).

Concentration	NR Activity (µMole/g LFW)	SOD (µMole/g LFW)	Catalase (µMole/g LFW)	Peroxidase (µMole/g LFW)	Polyphenol oxidase (µMole/g LFW)	Glutathione reductase (µMole/g LFW)
Control (H2O)	08.30 ± 0.131 (100)	07.91 ± 0.093 (100)	02.4 0 ± 0.038 (100)	07.23 ± 0.248 (100)	07.50 ± 0.061 (100)	10.73 ± 0.098 (100)
6 Mm Cu	04.86 ± 0.051 (59.13)	06.13 ± 0.157 (77.46)	04.02 ± 0.080 (167.6)	13.33 ± 0.226 (184.3)	11.61 ± 0.045 (154.7)	14.21 ± 0.129 (132.4)
2g *Dictyota*+ 6mM Co	05.50 ± 0.057 a (66.78)	06.30 ± 0.187 a (79.68)	03.80 ± 0.176 a (158.3)	10.99 ± 0.138 a (152)	10.11 ± 0.090 a (134.8)	13.33 ± 0.155 a (124.2)
4g *Dictyota*+ 6mM Co	06.00 ± 0.032 a (73.29)	06.54 ± 0.065 a (82.71)	03.02 ± 0.080 a (125.9)	08.72 ± 0.204 a (120.6)	09.13 ± 0.340 a (121.7)	12.91 ± 0.044 a (120.3)
6g *Dictyota*+ 6mM Co	07.75 ± 0.048 a (94.52)	07.06 ± 0.058 a (89.19)	02.75 ± 0.080 a (114.8)	07.64 ± 0.207 a (105.6)	08.48 ± 0.134 a (113)	11.65 ± 0.129 a (108.6)
8g *Dictyota*+ 6mM Co	08.13 ± 0.083 a (100.3)	08.01 ± 0.027 a (101.2)	02.43 ± 0.050 a (101.4)	07.18 ± 0.311 a (99.29)	07.54 ± 0.073 a (100.4)	10.86 ± 0.042 a (101.2)

Values are an average of six observations. Values in parenthesis are percentage activity with respect to control. Mean ± SE a – 2g, 4g, 6g and 8g *Ulva lactuca* treatment compared with control, Significance (P ≤ 0.05 – Turkey test).

Table 5 Atomic Absorbtion spectroscopic studies

SAMPLE	AAS in ppm
SAMPLE 1(Control)	0.8774
SAMPLE 2 (6 mM Co)	3.6240
SAMPLE 3 (2g *Dictyota* + 6 mM)	3.0761
SAMPLE 4 (4g *Dictyota* + 6 mM)	1.8046
SAMPLE 5 (6g *Dictyota* + 6 mM)	1.6269
SAMPLE 6 (8g *Dictyota* + 6 mM)	1.1216

Various species of marine alga were used as biosorbent for removal of heavy metals from environmental samples (Yu 1999; El-Sikaily 2007; Fagundes-Klen 2010; Laib 2011; Esmaeili 2011; Nessim 2011). In this study, *Dictyota dichotoma* cause metal absorbing properties. Removal of cadmium by biosorption onto the brown macroalgae *Dictyota dichotoma* Hannachi *et.al*. (2015), Maximum accumulation of Cr was recorded in *Dictyota* (6.272.2 mg/g) and the high uptake of metals in green algae (*Ulva lactuca* and *Enteromorpha intestinalis*) and brown algae (*Padina gymnospora* and *Dictyota bartayresiana*) suggested that these algae may be used as potential biomonitors for heavy metal pollution Sukalyan Chakraborty *et.al*. (2014).

In this biosorption study, the growth parameters are increased than cobalt control (6 mM) by the application of *Dictyota* with the respect of 2g, 4g, 6g and 8g with 6mM cobalt with comparison of *Dictyota dichotoma* exhibits promising effects on growth and yield characteristics of the test plant *Abelmoscus esculantus* Sasikumar *et.al*. (2011). The pigmental parameters are increased with cobalt metal control (6 mM) with the comparison with, Brown algae increase chlorophyll biosynthesis might have played a key role in the enhancement of growth and physiology and the results exhibited that shoot and root length, total fresh and dry weight, leaf area and the content of moisture, photosynthetic pigments, protein, amino acids, reducing sugar, ascorbic acid and nitrate reductase activity were found to be enhanced in the leaves of brinjal plants which received 1.5 % of *Stoechospermum marginatum* extracts and the content of total chlorophyll pigments, (77 %), protein (38 %), reducing sugar (201 %) and ascorbic acid (36 %) and nitrate reductase activity (159 %) was enhances when the brinjal plants were treated with SLE at 0.5, 1.0 and 1.5 % concentrations of brown algae, Sivasankari *et.al*. (2015).

In particular, accumulation of the amino acids alanine, glutamine, proline and serine in plants (both terrestrial and marine) has been associated with N uptake, with different amino acids responding to different N sources (Steward & Pollard, 1962; Silveira *et.al*. 1985; Lawlor *et.al*. 1987; Kiladze *et.al*. 1989; Di Martino Rigano *et.al*., 1992; Vona *et.al*. 1992; Heuer 1993).

The increase in photosynthetic pigments may be also be due to the presence of betaines Blunden *et.al*. (1997), increase in number and size of the chloroplast and better grana development (Atzmon and Van Staden 1994) in the SLE-treated plants. The biochemical contents are studied and are comparison with, brown algae increased the content of photosynthetic pigments, protein and total sugars in *Vigna radiata* Sivasankari *et.al*. (2006). The present data indicated that both POX and CAT activities were significantly

suppressed by pre-treatments with seaweed extract which was in agreement with results of Gharib *et.al*. (2014). *Dictyota dichotoma* under stress due to the epiphytism of L. lallemandii by means of the existence of oxidative lipid damage and the response of the CAT, SOD, GPX and GR activities. Silvia Tejada *et.al* (2014).

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How to cite this article:

Duraipandian M *et al*.2016, Improvement of Cobalt Chloride Caused Stress In Terms of Morphometric, Pigmental, Biochemical And Enzymatic Characteristics of Vigna Unguiculata (l.) by Biosorption Using Dictyota Dichotoma. *Int J Recent Sci Res.* 7(8), pp. 12941-12946.