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EFFECT OF *AZOTOBACTER* IN INTEGRATION WITH PAPER MILL EFFLUENT ON THE GROWTH OF *PENNISETUM AMERICANUM* (L)

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ABSTRACT

The effects of paper mill effluent on the growth and biochemical characteristics of 21 days old seedlings of *Pennisetum americanum* were studied with different concentrations (10%, 20%, 30%, 40%, and 50%) of the paper mill effluent. The effluent treatment on *Pennisetum* has caused a steep decline in its growth, pigment content, biochemical characteristics and enzyme activities with increase in the concentration of the effluent. On the other hand, the bioremediation treatment by various concentrations (1%, 2%, and 3%) of biofertilizer , *Azotobacter* were mixed separately with 30% (v/v) paper mill effluent individually on *Pennisetum* has brought out considerable increase on the growth and biochemical characteristics. The retarded growth of the paper mill effluent treated plants may be due the suspended and dissolved solids present in it. However, the addition of biofertilizers have mitigated the toxic effect on the effluent to some extent and instigated the growth parameters, pigment contents and biochemical characteristics and enzyme activities of *Pennisetum*. *A.chroococcum* exhibited better performance in reducing all the pollutants from the effluents than its counterparts. Bioremediation is more economical, acceptable and safe rather than any other physical and chemical process treatment because it removes the pollutant and it's toxicity through metabolic reactions mediated by the microorganisms. Since this methodology of employing natural potentials to deal with effluents' cleanup in an ecologically acceptable manner is attracting much attention now a days.

Key words: Effluent, *Azotobacter chroococcum.*

INTRODUCTION

Bioremediation is more economical, acceptable and safe rather than any other physical and chemical process treatment because it removes the polluent and its toxicity through metabolic reactions mediated by the microorganisms . Since this methodology of employing natural potentials to deal with effluents cleanup in an ecologically acceptable manner is attracting much attention now a days.. Pulp and paper industry is one of the largest industries in India and paper mill is one of the main industries in Sivakasi, industrial town of Tamil Nadu, India. Due to acute water scarcity in Sivakasi, the untreated effluents which are highly toxic to the growth of the plant system are being used as a source of irrigation for agriculture.

Under such system of irrigation, the vegetable crops like *P. americanum* has extensively cultivated in this area on large commercial scale are very badly affected. As this environmental problem has aroused serious concern, a safe and low cost Bioremedial treatment has been suggested as a remedial measure. In this context, the present investigation attempts to exploit *Azotobacter* for the remediation of paper mill effluent on the growth and biochemical profile of seedlings of *P. americanum*.

MATERIALS AND METHODS:

Healthy and viable seeds of *P. americanum* were surface sterilized with 0.1% mercuric chloride for one minute and washed with running tap water, followed by rinsing with distilled water. Various concentrations of paper mill effluent were prepared, such as 10%, 20% 30%, 40%, and 50% (v/v). The seeds were soaked in distilled water for two hours and allowed to grow in pots containing uniformly mixed red, black and sandy soil in 1:1:1 ratio. The experimental sets were kept in diffused light at room temperature. Seven days after sowing, the experimental plants were watered constantly every day with the respective concentration of the effluent (750 mL). The control sets were treated with tap water. Both experimental sets and control sets were maintained in triplicates. On the twenty first day both the sets of plants were taken for analysis.

BIOREMEDIATION

In the bioremediation treatment process, various concentrations (1%, 2%, and 3%) of *Azotobacter* were mixed separately with 30% (v/v) paper mill effluent, since that concentration was found to be optimum for *P. americanum*. The *P. americanum* seeds were sown to raise the seedlings in labeled pots. Control sets were maintained separately with tap water and the other with paper mill effluent (30% (v/v)). From the 7th day onwards the seedlings of *P. americanum* were treated with 750 mL of biofertilizer mixed effluent in the respective concentrations. On the 21st day, the seedlings were carefully removed from the soil without any damage to the root system and analysed the physical, biochemical and enzyme characteristics.

Growth and biochemical analysis

The growth parameters such as root length, shoot length, fresh weight, dry weight and leaf area were measured. The chlorophyll and carotenoids contents in the fresh leaf tissues were extracted and quantified using the formula suggested by Wellburn and Lichtenthaler (1984). Total sugar content (Jayaraman 1981), amino acid (Moore Stein *et al*., 1948), soluble leaf protein (Lowry *et al*., 1978), proline (Bates *et al*.,1973) *in vivo* nitrate reductase (Jaworski, 1971), peroxidase (Addy and Goodman, 1972), and catalase activity (Kar and Mishra, 1976) were also analyzed for both control and effluent treated seedlings.

RESULT AND DISCUSSION

Effect of growth and biochemical studies

The applications of various concentrations of paper mill effluent had brought about considerable reduction in the shoot length and root length, fresh weight, dry weight and leaf area of the experimental plants (Table 1). The total chlorophyll and carotenoids contents showed a declining trend with increasing concentrations of paper mill effluent (Table 2).

A pronounced reduction was observed in the biochemical characteristics of total soluble sugar and protein of *P. americanum*, after being treated with paper mill effluent. Whereas L- proline and leaf nitrate content were increased(Table 3). The differences in the biochemical contents are directly related to the differences in the metabolism, which in turn, depends on the enzyme activity. Hence, some major enzyme activities were analysed and the enzyme catalase and peroxidase activities were increased but nitrate reductase enzyme activity was significantly reduced (Table 4).

Table 1: Effect of Various Concentration of Paper Mill Effluent on the Growth Parameters of *P. americanum* (L.) Leeke

S.No	Parameters	Control	10% (v/v)	20% (v/v)	30% (v/v)	40% (v/v)	50% (v/v)
1.	**Shoot Length (cm)**	20.5 ± 0.145 (100)	19.2 ± 0.145** (93)	18.5 ± 0.176** (90)	17.2 ± 0.145** (83)	15.4 ± 0.145** (75)	14.5 ± 0.145** (70)
2.	**Root Length (cm)**	15.5 ± 0.152 (100)	14.4 ± 0.120** (93)	13.4 ± 0.120** (86)	11.4 ± 0.218** (73)	10.3 ± 0.115** (66)	8.2 ± 0.115** (52)
3.	**Fresh Weight (mg)**	0.018 ± 0.0003 (100)	0.016 ± 0.0005** (85)	0.015 ± 0008** (81)	0.014 ±0.001** (74)	0.013 ± 0.0008** (71)	0.012 ± 0.001** (64)
4.	**Dry Weight (mg)**	0.013 ± 0.0008 (100)	0.011 ± 0.0005** (82)	0.010 ± 0.0003** (80)	0.008± 0.0003** (65)	0.007 ± 0.0003** (55)	0.005 ± 0.0003** (42)
5.	**Leaf Area (cm^2)**	2.75 ± 0.020 (100)	2.64 ± 0.026** (96)	2.52 ± 0.011** (91)	2.23 ± 0.017** (81)	2.14 ± 0.005** (77)	1.94 ± 0.011** (70)

Values are an average of ten observations. Values in parentheses are percentage activity with respect to control. Mean ± Sε ** Significance at P<0.05 level

Table 2: Effect of Various Concentration of Paper Mill Effluent on the Photosynthetic Pigment content of *P. americanum* (L.) Leeke

S.No	Parameters	Control	10% (v/v)	20% (v/v)	30% (v/v)	40% (v/v)	50% (v/v)
1.	**Chlorophyll *a* (mg/gLFW)**	0.654 ± 0.002 (100)	0.593 ± 0.001** (90)	0.525 ± 0.001** (80)	0.495 ± 0.002** (75)	0.444 ± 0.001** (67)	0.395 ± 0.001** (60)
2.	**Chlorophyll *b* (mg/gLFW)**	0.625 ± 0.002 (100)	0.571 ± 0.0008** (91)	0.544 ± 0.001** (86)	0.473 ± 0.007** (75)	0.425 ± 0.002** (68)	0.378 ± 0.004** (60)
3.	**Total Chlorophyll (mg/gLFW)**	1.280 ± 0.062 (100)	1.165 ± 0.003** (95)	1.069 ± 0.002** (87)	0.969 ± 0.005** (79)	0.869 ± 0.001** (70)	0.773 ± 0.003** (63)
4.	**Carotenoids (mg/gLFW)**	3.194 ± 0.001 (100)	3.065 ± 0.002** (95)	3.817 ± 0.034** (88)	2.505 ± 0.031** (78)	2.004 ± 0.003** (62)	1.536 ± 0.022** (48)
5.	**Anthocyanin (mg/gLFW)**	4.856 ± 0.003 (100)	5.433 ± 0.088** (111)	5.783 ± 0.018** (119)	6.44 ± 0.017** (132)	7.16 ± 0.079** (147)	7.87 ± 0.027** (162)

Values are an average of five observations. Values in parentheses are percentage activity with respect to control. Mean ± Sε.(LFW –Leaf fresh weight)

Table 3: Effect of Various Concentration of Paper Mill Effluent on the Biochemical Characteristics of *P. americanum* (L.) Leeke

S.No	Parameters	Control	10% (v/v)	20% (v/v)	30% (v/v)	40% (v/v)	50% (v/v)
1.	**Glucose (mg/gLFW)**	33.56±0.202 (100)	28.53±0.176** (85)	24.53±0.185** (73)	21.6±0.173** (64)	19.5± 0.057** (58)	17.76±0.088** (52)
2.	**Protein (mg/gLFW)**	7.43 ± 0.120 (100)	6.53 ± 0.020** (87)	6.24 ± 0.023** (83)	6.12 ± 0.014** (82)	5.52 ± 0.014** (74)	4.24 ± 0.020** (57)
3.	**Starch (mg/gLFW)**	1.360 ± 0.008 (100)	1.023 ± 0.001** (75)	0.884 ± 0.001** (64)	0.785 ± 0.002** (57)	0.564 ± 0.002** (41)	0.427 ± 0.001** (31)
4.	**Amino acid (μmole/gLFW)**	10.4 ± 0.115 (100)	14.6 ± 0.145** (140)	16.5 ± 0.272** (158)	22.6 ± 0.145** (217)	25.6 ± 0.115** (246)	32.5 ± 0.173** (312)
5.	**Proline (mg/gLFW)**	0.597 ± 0.256 (100)	0.885 ± 0.002** (148)	0.947 ± 0.005** (158)	0.995 ± 0.001** (166)	1.054 ± 0.002** (176)	1.124 ± 0.001** (188)
6.	**Leaf nitrate (mg/gLFW)**	4.67 ± 0.008 (100)	5.16 ± 0.088** (110)	5.72 ± 0.034** (122)	6.78 ± 0.103** (145)	11.65 ± 0.017** (249)	17.56 ± 0.176** (375)

Values are an average of five observations. Values in parentheses are percentage activity with respect to control. Mean ± Sε ** Significance at P<0.05 level . (LFW –Leaf fresh weight)

Table 4: Effect of Various Concentration of Paper Mill Effluent on Enzyme Activities of *P. americanum* (L.) Leeke

S.No	Parameters	Control	10% (v/v)	20% (v/v)	30% (v/v)	40% (v/v)	50% (v/v)
1.	**Nitrate reductase (µmole/gLFW)**	12.85± 0.011 (100)	10.83± 0.008** (84)	9.34± 0.008** (72)	7.84± 0.005** (61)	6.35± 0.011** (49)	4.66± 0.008** (36)
2.	**Catalase (µmole/gLFW)**	2.8±0.057 (100)	3.36±0.218** (120)	3.80± 0.115** (135)	6.20± 0.152** (221)	8.60± 0.152** (307)	11.50± 0.173** (410)
3.	**Peroxidase (µmole/gLFW)**	0.004±0.0008 (100)	0.009± 0.0005** (209)	0.012±.0008** (294)	0.015± 0.0003** (356)	0.019± 0.0008** (457)	0.026± 0.0005** (604)

Values are an average of five observations. Values in parentheses are percentage activity with respect to control. Mean ± Sε ** Significance at P<0.05 level . (LFW –Leaf fresh weight)

Bioremediation Studies

Azotobacter causes mineralization of fixed phosphate of soil and thus increase uptake of phosphate in plants. A freshly isolated strain of *A. chroococcum* fixed nitrogen more efficiently when grown in association with another soil bacterium (Parker, 2003).

Addition of the various concentrations of *Azotobacter* with paper mill effluent caused a significant increase on the growth of the seedlings. With the addition of 3% *Azotobacter* the increase in the shoot length was 92%, higher than when they were treated with paper mill effluent alone. Similar increasing trend in the root length was noticed with various concentrations of *Azotobacter* integrated with paper mill effluent. There was a gradual increase in the root length with the increasing concentrations of *Azotobacter*. The use of these nitrogen fixing bacterias as a foliar, particularly the *Azotobacter*, increased mulberry leaf production (Sudhakar *et al*., 2000). *Azotobacter* inoculation in combination with triptophan gave maximum length and weight of maize roots and shoots than control (Zahir Ahmad Zahir *et al*., 2000).

The application of *Azotobacter* integrated with paper mill effluent showed better results in the fresh weight, dry weight and leaf area than the control sets treated with paper mill effluent alone (Table 5).

Photosynthetic Pigment Content

The photosynthetic pigments such as chlorophyll *a*, *b*, total chlorophyll and carotenoid contents were increased in the plants with increasing concentrations of *Azotobacter*. The maximum increase in total chlorophyll content was about 95% than they were treated with paper mill effluent alone.

A reduction in anthocyanin content was observed in the plants when they were treated with *Azotobacter* . (Table 6). Increased population of N_2 fixing bacteria in the rhizosphere led to increase of chlorophyll pigments followed by increase of total soluble sugar. It was reported that mineral, organic fertilizers and *Azotobacter* applied alone and in various combinations significantly improved the yield components and N uptake by wheat crop when compared to control (Mohammad Idris, 2003).

Biochemical Characteristics

The bioremediated paper mill effluent showed significant increase in total soluble sugar and leaf protein contents. The maximum increase in total soluble sugar was 83% for *Azotobacter* than when they were treated with paper mill effluent alone. *Azotobacter* in the presence of 75 kg N ha-1 were found to have significantly improved the growth and yield parameters, nitrogen content in the *Allium cepa* bulb and soil available nitrogen (Tesfaye Balem *et al*., 2007).

The protein content was also increased after treatment with biofertilizer integrated paper mill effluent. It was at the rate of 91% for *Azotobacter* than the plants were treated with effluent alone. Accumulation of soluble leaf protein was an indication of high metabolic status of Nitrogen. . The amendment and inoculum improved the quality of treated Cambu plants by recovering from the stress and hence the reduction of proline to the maximum. Stewart and Lee (1974) reported that the accumulation of proline was an adaptive response to stress. Decreasing content of anthocyanin subsequently decreased the amount of phenol in biotreated plant. Anthocyanin acts as a regulator for the synthesis of phenol (Zamprometov and Zagoskina, 1987).

Amino acid, proline and leaf nitrate contents were decreased with increasing concentrations of *Azotobacter* integrated with paper mill effluent (Table 7). *Azotobacter* is tolerant to high salts. Biofertilizers improve soil fertility and enhance nutrient uptake and water uptake in deficient soils, thereby aiding in better establishment of plants.

Table 5: Effect of Paper Mill Effluent with *Azotobacter* on the Growth Parameters of *P. americanum* (L.) Leeke

S. No.	Parameters	Control (water)	Effluent 30% v/v)	1% (w/v)	2% (w/v)	3% (w/v)
1.	Shoot Length (cm)	20.53 ± 0.145 (100)	17.23 ± 0.145** (83)	17.66 ± 0.088** (86)	18.36 ± 0.120** (89)	18.90 ± 0.208** (92)
2.	Root Length (cm)	15.50 ± 0.152 (100)	11.46 ± 0.218** (73)	13.36 ± 0.126** (86)	13.83 ± 0.087** (89)	14.03 ± 0.088** (90)
3.	Fresh Weight (mg)	0.018 ± 0.0003 (100)	0.014 ± 0.001** (74)	0.016 ± 0.0006** (89)	0.017 ± 0.0003** (89)	0.018±0.0006** (90)
4.	Dry Weight (mg)	0.013 ± 0.0008 (100)	0.008 ± 0.0003** (65)	0.009 ± 0.0006** (70)	0.011 ± 0.0005** (82)	0.012 ± 0.0008** (87)
5.	Leaf Area (cm^2)	2.75 ± 0.020 (100)	2.23 ± 0.017** (81)	2.34 ± 0.026** (85)	2.41 ± 0.023** (87)	2.50 ± 0.031** (91)

Values are an average of ten observations. Values in parentheses are percentage activity with respect to control. Mean ± Sε ** Significance at P<0.05 level . (LFW –Leaf fresh weight)

Table 6: Effect of Paper Mill Effluent with *Azotobacter* on the Photosynthetic Pigment content of *P. americanum* (L.) Leeke

S. No.	Parameters	Control (water)	Effluent 30% (v/v)	1% (w/v)	2% (w/v)	3% (w/v)
1.	Chlorophyll *a* (mg/gLFW)	0.654 ± 0.017 (100)	0.495 ± 0.002** (75)	0.506 ± 0.0018** (77)	0.549 ± 0.001** (83)	0.600 ± 0.006** (91)
2.	Chlorophyll *b* (mg/gLFW)	0.625 ± 0.002 (100)	0.473 ± 0.007** (75)	0.507 ± 0.001** (81)	0.528 ± 0.001** (84)	0.566 ± 0.002** (90)
3.	Total chlorophyll (mg/gLFW)	1.280 ± 0.002 (100)	0.969 ± 0.005** (79)	1.013 ± 0.0008** (82)	1.077 ± 0.002** (87)	1.16 ± 0.005** (95)
4.	Carotenoids (mg/gLFW)	3.194 ± 0.001 (100)	2.505 ± 0.031** (78)	2.606 ± 0.002** (81)	2.73 ± 0.001** (85)	2.87 ± 0.010** (90)
5.	Anthocyanin (mg/gLFW)	4.85 ± 0.003 (100)	6.44 ± 0.174** (132)	5.86 ± 0.064** (120)	5.36 ± 0.014** (110)	5.14 ± 0.029** (105)

Values are an average of five observations. Values in parentheses are percentage activity with respect to control. Mean ± Sɛ.(LFW –Leaf fresh weight)

Table 7: Effect of Paper Mill Effluent with *Azotobacter* on the Biochemical Characteristics of *P. americanum* (L.) Leeke

S. No.	Parameters	Control (water)	Effluent 30% (v/v)	1% (w/v)	2% (w/v)	3% (w/v)
1.	**Glucose (mg/gLFW)**	33.56 ± 0.202 (100)	21.60 ± 0.173** (64)	23.50 ± 0.208** (70)	25.43 ± 0.240** (75)	27.90 ± 0.378** (83)
2.	**Protein (mg/gLFW)**	7.43 ± 0.120 (100)	6.12 ± 0.014** (82)	6.36 ± 0.013** (85)	6.62 ± 0.021** (89)	6.80 ± 0.152** (91)
3.	**Starch (mg/gLFW)**	1.36 ± 0.0008 (100)	0.78 ± 0.002** (57)	0.82 ± 0.004** (60)	0.84 ± 0.001** (62)	0.89 ± 0.001** (85)
4.	**Amino Acid (µmole/gLFW)**	10.40 ± 0.115 (100)	22.66 ± 0.145** (217)	20.63 ± 0.317** (198)	18.43 ± 0.120** (177)	15.20 ± 0.351** (146)
5.	**Proline (mg/gLFW)**	0.59 ± 0.256 (100)	0.99 ± 0.001** (166)	0.95 ± 0.001** (159)	0.93 ± 0.001** (156)	0.91 ± 0.001** (153)
6.	**Leaf Nitrate (mg/gLFW)**	4.67 ± 0.008 (100)	6.78 ± 0.103** (145)	6.63 ± 0.088** (141)	6.23 ± 0.033** (133)	5.43 ± 0.120** (116)

Values are an average of five observations. Values in parentheses are percentage activity with respect to control. Mean ± Sε ** Significance at P<0.05 level (LFW –Leaf fresh weight)

Table 8: Effect of Paper Mill Effluent with *Azotobacter* on the Enzyme Activities of *P. americanum* (L.) Leeke

S. No.	Parameters	Control (water)	Effluent 30% (v/v)	1% (w/v)	2% (w/v)	3% (w/v)
1.	**Nitrate reductase (µmole/gLFW)**	12.85 ± 0.0115 (100)	7.84 ± 0.005** (61)	8.29 ± 0.040** (64)	9.30 ± 0.027** (72)	10.32 ± 0.096** (80)
2.	**Catalase (µmole/gLFW)**	2.80 ± 0.057 (100)	6.20 ± 0.152** (221)	5.70 ± 0.057** (203)	4.46 ± 0.088** (159)	3.43 ± 0.120** (122)
3.	**Peroxidase (µmole/gLFW)**	0.0043 ± 0.0008 (100)	0.0153 ± 0.0003** (356)	0.0130 ± 0.0005** (302)	0.0103 ± 0.0003** (240)	0.0087 ± 0.0003** (201)

Values are an average of five observations. Values in parentheses are percentage activity with respect to control. Mean ± Sε Significance at P<0.05 level. (LFW –Leaf fresh weight)**

Enzyme Activities

The activities of catalase and peroxidase were found to be decreased with the increase in concentrations of the biofertilizer (Table 8). The peroxidase enzyme also followed a negative trend as catalase, when the biofertilizers integrated with paper mill effluent treated on Cambu . However, *in vivo* nitrate reductase activity was increased with the increase in the concentration of bioremediated paper mill effluent.

CONCLUSION

It could reasonably be said that the application of *Azotobacter* for bioremediation of paper mill effluent increased the growth and biochemical parameters of *P. americanum* when it was compared with control plants treated with water and effluent alone.

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