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Research Article

Peroxidase as a metric of stress tolerance and invasive potential of alligator weed (*Alternanthera philoxeroides*) growing in aquatic habitats

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Abstract

An attempt was made to understand the potential of *Alternanthera philoxeroides*, alligator weed to adapt to diverse conditions present in pond ecosystems, through a correlative investigation of its natural growth pattern and peroxidase level. Eleven ponds were graded into two subjective categories: "*A. philoxeroides* Infestation" (High, Medium, Low) and "Level of Pollution" (High and Low), to test for difference in mean peroxidase concentration in *A. philoxeroides* populations. Significant changes in mean peroxidase concentration in *A. philoxeroides* were found in ponds categorized on the basis of level of pollution, indicating the adaptability of this plant to propagate under pollution stress. On the other hand, there was no significant change in mean peroxidase concentration for plants growing in ponds categorized on the basis of infestation showed that dense, vegetative proliferation caused no stress in *A. philoxeroides.* An efficient method of assaying peroxidase in *A. philoxeroides,* under field conditions, using the best suited leaf group (Tips, Tips + 1st leaf pair", 2nd leaf pair) was also explored. "Tips + 1st leaf pair" proved to be a better sample than mature leaves for estimation of peroxidase concentration in *A. philoxeroides.*

Key words: *Alternanthera philoxeroides*; peroxidase; stress; plant invasion, adaptation

Introduction

Biological invasions by non-native species are one of the worst ecological components of global environmental change. Once established, aquatic invasive plant species can have dramatic effects on the native ecosystem (Homans and Smith 2011). *Alternanthera philoxeroides* (Martius) Griseb (Amaranthaceae), alligator weed, an invasive perennial wetland herb originating from South America (Maddox 1968), has many attributes of a successful invasive weed such as rapid growth and vegetative propagation (Tao et al. 2009; Wang et al. 2009) as well as a broad ecological amplitude (Geng et al. 2007). This amphibious clonal plant has invaded many ecosystems worldwide (Julien et al. 1995), including riparian zones (Pan et al. 2006) and agricultural lands (Spencer and Coulson 1976), and is regarded as one of the worst weeds of the world (http://www.waikatoregion.nz).

The presence of *A. philoxeroides* in India was first reported by Maheshwari (1965) mostly in lakes and Cook (1996) reported it in ponds. There are scanty reports of this plant during the twentieth century but there seems to be a flood of reports from all over India since the last decade, reviewed by Chatterjee and Dewanji (2010). These reports show that *A. philoxeroides* has spread across climatologically diverse regions of India, like the Himalayan foothills (Negi and Hajra 2007) and mountainous states of Jammu and Kashmir (Masoodi and Khan 2012), wet rain-fed regions of North-East India (Jain et al. 2007; Singh et al. 2010), fertile Eastern Plains (Paria and Mukherjee 1981), drier plains of Central India (Singh and Pandey 1998), Western India (Wagh et al. 1995), tropical Southern India (Reddy et al. 2008) and even in coastal regions like Andaman Islands (Reddy and Raju 2005), thus indicating its ability to proliferate under diverse phytogeographical regions. The presence

of large areas of inland aquatic bodies and wetlands in India (Prasad et al. 2004) serves as a potential habitat for the invasive *A. philoxeroides* (Chatterjee and Dewanji 2010). Once established, *A. philoxeroides* is extremely hard to control and eradication is very expensive (Jia et al. 2009; Sainty et al. 1997), thereby creating an urgent need to understand the present status of invasion in this region. Invasive plants posses certain characteristics which make them more competent to adapt and propagate in newer environments/ habitats and understanding these mechanisms is crucial for managing invasive plant species (Mack et al. 2000).

The adaptation of *A. philoxeroides* to these diverse environmental conditions might be due to its inherent potential of combating stress by up-regulation of the defence mechanism of the plant. A multitude of abiotic stresses such as drought, salinity, extreme temperature and chemical toxicity pose serious threats to plants and cause rapid and excessive accumulation of reactive oxygen species, leading to oxidative stress. Peroxidase is one of the most responsive enzymes to environmental stresses as its activity has been associated with a wide range of physiological processes and can be readily monitored in crude extracts of plant tissue that readily support spectrophotometric and electrophoretic investigations (Quesada et al. 1992).

Considerable efforts have been directed towards the establishment of using enhanced peroxidise activity in plants under a variety of stresses, such as salinity (Rout and Shaw 2001), drought (Gao et al. 2008) and herbicides (Sprecher et al. 1993). Changes in peroxidase enzyme activity and its association with environmental stress in both aquatic (Hanfeng et al. 2010; Nayak et al. 2010) and terrestrial plants (Markkola et al. 1990; Fekete et al. 2002; Aki et al. 2009) have also been reported. But most studies on peroxidase enzyme assays in *A. philoxeroides* have been done either with plants (Ding et al. 2007; Gao et al. 2008; Santra et al. 2003; Xu et al. 2011a) or with callus (Xu et al. 2011b) or cell cultures (Balagtas-Burrow et al. 1993) under lab-controlled conditions. While lab bioassays may be useful as initial screening mechanisms by establishing direct physiological responses to different stressors, Schindler (1987) stressed the need for more comprehensive studies, in natural systems, for estimating the effects of stressors which could eventually form the basis of management procedures, if and when required.

This study was, therefore, undertaken to establish stress levels in *A. philoxeroides* under field conditions. We hypothesise that changes in peroxidase concentration, which has been associated with environmental stress, can be used to relate stress-adaptation and indicate the invasive potential of *Alternanthera philoxeroides*, in pond ecosystems. The following were the two objectives of this study:

(1) To undertake a baseline study of peroxidase concentration in *A. philoxeroides* growing along the littoral zone in different ponds and try to correlate peroxidase levels with stress tolerance of *A. philoxeroides* in terms of its strand-density (quantified through degree of "*A. philoxeroides* infestation") and eutrophic stress (categorized through "Level of Pollution") of the ponds.

(2) To assay the comparative enzyme extraction efficiency of peroxidase concentration amongst three sets of leaf sample groups of *A. philoxeroides*, since quite often it is not always feasible to get adequate amount of fresh young leaves for enzyme quantification.

Materials and methods

Sampling sites

Samples of *A. philoxeroides* and water were collected from eleven ponds located in Baranagar, Kolkata, India. The geographical location of ponds, local names, sizes, major associated flora, and gradation on the basis of extent of *A. philoxeroides* infestation and level of pollution based on water quality are given in Table 1A.

The ponds were selected such that they had varying levels of *A. philoxeroides* infestation in them. To categorize infestation, percentage cover of *A. philoxeroides* in each pond was recorded by floating a $1m^2$ quadrat and based on estimated *A. philoxeroides* cover percent in the ponds, three categories of "*A. philoxeroides* infestation" were chosen, namely "High" (>75%) under which there were 5 ponds, "Medium" (20% - 75%) which had 4 ponds and "Low" (<20%) with only 2 ponds (Table 1A). Infestation levels from Low to High can also be visualized from Figure 1 where six of the eleven study ponds are shown e.g., Pond 1 (Figure 1 A), a relatively clean-pond with limited access while at the other extreme was Pond 10 (Figure 1 E), a highly polluted pond located in the vicinity of some factories with maximum influx of factory-wastes.

Figure 1. Six of the study ponds showing different degrees of *Alternanthera philoxeroides* infestation and pollution influx. A: Pond 1 (Amrapali), B: Pond 2 (Dakshineswar), C: Pond 3 (Bidyatan Sarani), D: Pond 8 (Kancher Mandir – 1), E: Pond 10 (Belghoria), F: Pond 11 (Tantipara). Red outlined areas in the photographs show regions of *A. philoxeroides* infestation (Photographs by Anindita Chatterjee).

Water quality estimation

Water samples from the ponds were analyzed for some important water quality parameters to get an idea about the pollution level present in these ponds. Using a hand-held probe (Model HQ40d, HACH Company, USA), in situ measurements of conductivity (Hach Method 8160) (Standard method 2510-B) and pH (Hach Method 8156 pH Meter/Electrode Electrode Method) (USEPA method 150.1) were made. Dissolved Oxygen was measured using Portable Luminescent Dissolved Oxygen (LDO) meter (Model HQ30d, probe LDO 101, HACH Company, USA) (Hach Method 10360 D) (USEPA 40 CFR 136). Water samples were brought to the laboratory where turbidity was assessed using a turbidimeter (Model 2100P, HACH Company, USA) (EPA method 180.1), total Phosphorus was determined by the ascorbic acid method (APHA, 1998) and COD (chemical oxygen demand) was measured by photometric determination of chromate consumption by the organic compounds, after digestion in concentrated sulphuric acid solution at 148°C for 2 h by means of COD Cell Test by Merck Spectroquant (EPA method 410.4).

Each water quality parameter tested was classified into two groups denoting "Level of Pollution" on the basis of high and low values as reported in Table 1B. Final cut offs for the two categories of ponds, namely "High" and "Low", were based on standard criteria for surface water quality as per Central Pollution Control Board, India (http://www.cpcb.nic.in) and Environmental Protection Agency, U.S.A. (http://www.epa.gov) guidelines for the three basic parameters - pH, DO and COD. Out of the eleven ponds studied, 5 ponds could be classified under the "High" category while 6 ponds could be put under the "Low" category as can be seen from Table 1. Additionally, it was observed that the "High" ponds had an external influx of industrial and/or domestic pollutants into them while the "Low" ponds were generally located within protected premises and were subjected to limited public access/use.

Table 1A. Descriptives of the ponds selected for the study.

Sl. No.	Pond Name	Latitude Longitude	Size of Pond *	Major Associated Flora	*A. philoxeroides* Infestation **	Level of Pollution #
1	AMRAPALI	22°38'47.21"N 88°22'37.08"E	Medium	*Vallisneria spiralis*	Low	Low
2	DAKHINESWAR	22°39'20.57"N 88°22'15.59"E	Large	*Pistia stratiotes* *Spirodela polyrrhiza*	Medium	Low
3	BIDYATAN SARANI	22°38'58.67"N 88°22'3.53"E	Medium	-	Medium	Low
4	BENGAL IMMUNITY CLUB	22°37'56.43"N 88°22'31.67"E	Medium	*Lemna aequinoctialis* *Spirodela polyrrhiza*	Medium	Low
5	DN 9/1 BUS STAND	22°39'5.23"N 88°21'47.13"E	Large	*Eichhornia crassipes* *Lemna aequinoctialis* *Spirodela polyrrhiza*	Medium	High
6	BARANAGAR BAZAR	22°37'58.69"N 88°22'13.63"E	Large	*Lemna aequinoctialis* *Spirodela polyrrhiza*	High	Low
7	PRAKASH KAUR	22°40'2.63"N 88°22'30.43"E	Small	*Ipomoea aquatica* *Lemna aequinoctialis*	Low	Low
8	KANCHER MANDIR-1	22°38'22.34"N 88°21'45.75"E	Small	*Lemna aequinoctialis* *Spirodela polyrrhiza*	High	High
9	KANCHER MANDIR-2	22°38'22.41"N 88°21'46.76"E	Small	*Eichhornia crassipes* *Lemna aequinoctialis* *Spirodela polyrrhiza*	High	High
10	BELGHORIA	22°39'34.41"N 88°22'45.88"E	Medium	*Mikania scandens* *Eichhornia crassipes* *Ipomoea aquatica* *Lemna aequinoctialis*	High	High
11	TANTIPARA	22°38'33.10"N 88°22'0.51"E	Small	*Spirodela polyrrhiza* *Mikania micrantha* *Colocasia esculenta*	High	High

* Large: (2000 – 5000 sq. m); Medium: (1000 – 2000 sq.m); Small: (500 – 1000 sq.m)
** High: (>75% Cover); Medium: (20% - 75% Cover); Low: (<20% cover)
# Low and High Level of Pollution based on criteria given in Table 1B

Table 1B. Categorisation of the study ponds on the basis of "Level of Pollution":

Level of Pollution	pH	Conductivity (µS / cm)	Turbidity (NTU)	Dissolved Oxygen (mg/L)	Total Phosphorus (mg/L)	Chemical Oxygen Demand (mg/L)
Low	6.5 – 7.5	< 1000	< 10	> 6.0	< 5	< 5
High	<6.5	> 1000	> 25	< 3.5	> 5	> 50

Plant sampling for peroxidise estimation

Sampling was conducted in two phases, between the months of September to October in 2010 and December 2010 to January 2011 since the plant are reported to grow luxuriantly during the post monsoon (Sept-Oct) and winter (Nov-Jan) months in this region (Mukhopadhyay and Dewanji 2004). Littoral rooted and floating-matted *A. philoxeroides* were sampled, as per availability, between 9:00 a.m to 12:00 noon.

It was not always be possible to sample 200-400 mg of fresh leaf tips/buds, required for enzyme quantification, due to low population size at sites/ the minute size of leaf-tips (leaf buds) (0.3 – 0.8 cm) / herbivory and/or insect attacks. It was, therefore, thought appropriate to collect samples from the following three leaf groups in order to determine which among the three leaf groups was best suited for enzyme extraction:

i. The young minute apical tips (leaf buds) (designated as T) (0.3 – 0.8 cm)

ii. The apical tips (leaf buds) plus the next young leaf-pair (designated as A) (0.8 – 1.5 cm)
iii. The second nodal leaf-pair (designated as B) (1.5 – 2.0 cm)

The first two sets (T and A) comprised of 41 harvests each, considered as replicates, from eleven ponds. Simultaneously, as an additional set of experiment, the 2nd nodal leaves of 35 samples (B), each considered as replicates, selected from 10 ponds, were harvested.

After harvesting, each set of leaf-samples were immediately packed in aluminium foil, labelled and stored in ice for transport to the laboratory. Once in the laboratory, they were immediately stored in at -20°C temperature in a freezer. The samples were analysed for estimation of peroxidase concentration on the same day of sampling.

Enzyme extraction and quantification

Peroxidase was quantified according to Shannon et al. (1966) with some minor modifications. In the laboratory, a 200 mg fresh leaf sample was taken and macerated in 1.5 ml 0.9% KCl under ice-cold conditions. The homogenate was centrifuged at 12,000 rpm at -2°C for 15 minutes (Remi Centrifuge, Model no. C-24). The supernatants were analysed for enzyme activity. The reaction mixture contained 2.5 ml Acetate Buffer, 0.1 ml 3% H_2O_2 (substrate), 0.1 ml o-Dianisidine dihydrochloride (Sigma-Aldrich, D3252-25G) (dye) and 0.1 ml prepared enzyme sample. After incubating for 30 min at room temperature, the reaction was stopped using 5N H_2SO_4. A standard curve of Horse-Radish Peroxidase (HRP) (Sigma, P6782-5MG) enzyme was prepared by using different aliquots (20, 40, 60, 80, 100 µg/ml) of the stock HRP enzyme (1mg/ml) and subjecting it to the same treatments. The sample absorbance was read at 460 nm against a blank in the spectrophotometer (Thermo Electron Corporation Helios γ). The peroxidase enzyme concentrations of all groups {(T), (A) and (B)}, were calculated and the concentration was reported as µg/gm fresh weight of leaf (henceforth written as µg/gm F.W.).

Statistical analysis

Exploratory data analysis was done and the descriptive statistics were obtained for the three leaf groups and the different ponds studied. Since the observations from the three leaf groups from a particular pond are likely to be dependent, hence, in order to compare any two of them, a paired sample t-test was considered appropriate based on paired observations from the eleven ponds, assumptions of normality and homogeneity of variance were checked with Kolmogorov-Smirnov's and Levene's tests, respectively. For this purpose, since there were varying number of observations from the different ponds, two observations from each pond for each type of leaf group were randomly selected to carry out the paired analysis based on their means. This ensured homogeneity of variance of the observed mean differences over the eleven ponds. Whenever, there were more than two observations (i.e., in case of Pond No's 1, 10 and 11), two observations were chosen randomly for the analysis. Out of are nearly 13,000 such possible combinations for testing multiple random pairs, 100 such random combinations was rechecked to verify the results of the analysis. ANOVA (using SPSS 16.0) was performed for the three leaf groups to check for difference between ponds graded as per their extent of infestation as well as for ponds graded as per their level of pollution. As in the case of paired t-test, the means of the two randomly selected observations for each pond were also used for ANOVA to ensure homoscedascity.

Results

Associated flora

From Table 1**,** it can be seen that only Pond 3 was devoid of any other macrophyte growth while the presence of submerged vegetation (*Vallisneria spiralis)* was noted in only one pond (Pond 1). Duckweeds (*Lemna/Spirodela* spp.) were the most common floating species observed in nine of the eleven ponds. *Pistia stratiotes* (Pond 2), *Ipomoea aquatica* (Ponds 7 and 11), *Colocasia esculenta* (Pond 10) and *Mikenia micrantha* (Ponds 9 and 10) were the other associated species observed.

Peroxide concentration

The mean as well as the maximum and minimum peroxide concentration (µg/gm FW) of the three leaf groups {(T), (A) and (B)} in the eleven ponds in Baranagar, Kolkata is reported in Table 2. A high degree of variation in the range values (maximum and minimum) for peroxidase concentration can be seen between groups in

individual ponds. The lowest value of the range for mean peroxidase concentrations was found for Group T in Pond 7 (6.71 µg/gm FW), for Group A in Pond 2 (6.72 µg/gm F.W.) and for Group B in Pond 3 (1.31 µg/gm F.W.). On the other hand, Pond 10 (incidentally the most polluted due to influx of factory wastes (Figure 1)) showed a consistently large variation (> 270 µg/gm FW) for all groups. High mean peroxidase values (>500 µg/gm FW) was also noted in all the leaf groups in the same pond (Pond 10).

Exploratory data analysis of peroxidase enzyme concentration (µg/gm FW) for each of the three leaf groups based on individual observations from all ponds was also done to get an idea about the nature of the data and the resulting box-plots can be seen in Figure 2. The results show that median values for peroxidase concentration of leaf group B were lower than that of groups A and T. The mean values also listed in the figure show the same trend. The variability of the peroxide concentrations in the three groups of leaf samples seems to be similar.

The results of the three possible comparisons of the leaf groups through paired t-test are given in Table 3. While there is strong significant evidence of higher means for mean peroxidase concentrations in both A ($p<0.000$) and T ($p<0.000$) samples compared to that of group B, the means of A and T groups were not statistically different ($p=0.284$).

Of the 100 such random combinations that were also considered for multiple testing for the paired t-test analysis, although there was a slight different in p values but the qualitative inference remained the same namely, in all cases the null hypotheses was rejected which is similar to the results reported for the given random combination presented in Table 3. This conforms to the findings in the box plots (Figure 2) and bar diagram (Figure 3) which shows the mean peroxidase concentrations for each leaf group based on two randomly selected observations from each of the eleven ponds. The figure distinctly shows evidence in favour of some pond-to-pond variation in mean peroxidase levels. Ponds 5, 8, 10, and 11 show higher mean peroxidase values compared to others and there are some ponds (Ponds 1, 2, 6 and 7) which show lower values. This difference suggests the existence of other factors that might be responsible for this pond-to-pond variation. Thus, ponds were divided into two categories (namely "*A. philoxeroides* infestation" and "Level of Pollution") as mentioned in Table 1 and ANOVA was done to test equality of means in different categories for a particular leaf group (e.g., A, T and B) based on the observed means for peroxidase concentration (of randomly selected two observations) ensuring homoscedasticity. The results are reported in Table 4.

From the table it is evident that there is no significant difference in the peroxidase concentration for each leaf group (T, A and B) on the basis of *A. philoxeroides* infestation categories of the study ponds. However, significant differences were observed for the same when the ponds were categorized on the basis of influx of pollution, with T having the highest significance value ($p=0.005$) amongst the three leaf groups (Table 4). Although there is a significant difference in mean peroxidase concentration between the two "level of pollution" categories (Low and High), a paired sample t-test was again carried out to check for differences within each of the two categories and results are reported in Table 5. No significant difference between the leaf-groups T and A can be seen which confirms with the results of the paired t-test reported earlier (Table 3).

Discussions

Enzyme extraction efficiency amongst leaf groups of A. philoxeroides

Different plant parts have been reported to contain different concentrations of cellular stress enzyme and such variations in antioxidant potentials and peroxidase concentration have been observed in root, stem, leaf, flowers and pods in two varieties of *Catharanthus roseus* (Jaleel et al. 2008). Most studies use young plant parts like apical shoots or young leaves (Roy et al. 1992; Cakmak and Marschner 1992) or seedlings (Verma and Dubey 2003) for enzyme quantification although there are reports on the use of roots also in laboratory controlled experiments (Santra et al. 2003, Gao et al. 2008). However, young, apical leaves were used for enzyme quantification in this study under the assumption that they contain higher amounts of active enzyme molecules and lesser amounts of other interfering compounds. Moreover, under field conditions, collection of leaves is more convenient compared to roots, which may get destroyed during removal thus hampering enzyme extraction. Zou et al. (2012) also reported that leaves of aquatic *A. philoxeroides* had higher peroxidase than stem or roots. Three

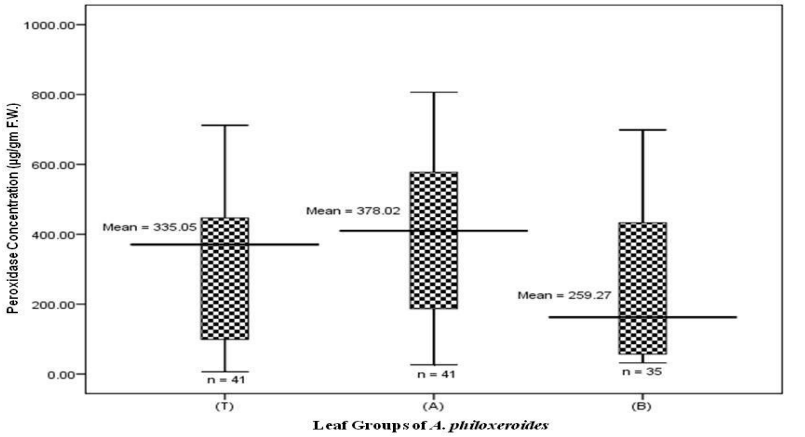

Figure 2. Box-Plot showing the peroxidase concentrations in three leaf groups of *A. philoxeroides* (across all the eleven study ponds pooled together).

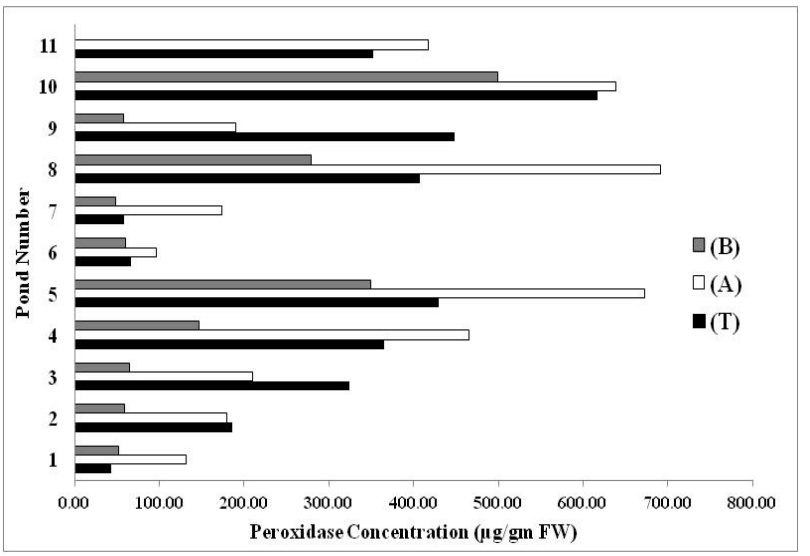

Figure 3. Comparison of peroxidase concentration values for leaf-groups (T, A and B) for 11 ponds (taking randomly selected 2-data-points for each pond).

leaf groups were tested for determination of the best suited leaf group for enzyme quantification.

Amongst the three leaf groups of *A. philoxeroides*, as is evident from Figure 2, the highest mean peroxidase concentration was found in leaf group A (378.02 µg/gm F.W.) followed by leaf group T (335.05 µg/gm F.W.) and leaf group B (259.27 µg/gm F.W.). Paired-sample t-test between leaf groups showed significant difference in the mean peroxidase values of leaf group B with both A and T ($p < 0.010$) (Table 3). Relatively older leaves, in Leaf group B, containing a lesser quantity of peroxidase could probably account for this difference. As enzyme molecules are highly temperature sensitive, degradation of enzymes fractions are possible during field sampling and transport (Malik and Singh 1980). Due to their very small size (0.3 – 0.8 cm), the younger tips (leaf group T) of the plants are more likely to be subjected to heat-mediated enzyme degradation during harvesting. Thus, when the next leaf pair of comparatively bigger size (0.8 – 1.5 cm) are also included with the tips (leaf group A), collection of the required amount of samples for the enzyme assay (Shannon et al. 1966) can be much faster, thereby, reducing the chance of heat-mediated enzyme degradation.

Differences in mean peroxidase concentration between leaf group A and T in some ponds (e.g., Ponds 3 and 9 had greater peroxidase values for group T as compared to that of group A) were evident from Figure 3. However, no significant difference ($p = 0.282$) in the mean peroxidase

Table 2. Pond-wise Mean Peroxidase Concentration (µg/gm F.W.) of the three test groups (Tips=T; Tips & 1st Leaf = A) and the 2nd Nodal Leaf (B):

Pond No.	N	Tips (T) (µg/gm F.W.)			N	Tips & Leaf (A) (µg/gm F.W.)			N	2nd Nodal Leaf (B) (µg/gm F.W.)		
		Mean ± S.E.	Min	Max		Mean ± S.E.	Min	Max		Mean ± S.E.	Min	Max
1	9	84.41 ± 16.92	6.49	162.95	9	120.24 ± 34.10	26.42	289.00	6	45.81 ± 5.90	32.18	71.00
2	2	185.80 ± 17.56	168.24	203.36	2	180.16 ± 11.61	168.55	191.76	2	58.93 ± 1.53	57.40	60.46
3	2	324.59 ± 18.36	306.23	342.95	2	210.17 ± 8.86	201.31	219.02	2	64.92 ± 0.66	64.26	65.57
4	2	365.57 ± 15.41	350.16	380.98	2	464.92 ± 112.13	352.79	577.05	2	147.22 ± 17.37	129.84	164.59
5	2	429.18 ± 17.38	411.80	446.56	2	673.12 ± 12.14	660.98	685.25	2	349.19 ± 6.89	342.30	356.07
6	2	66.41 ± 4.12	62.29	70.53	2	196.19 ± 27.03	169.16	223.21	2	60.61 ± 5.95	54.66	66.56
7	2	58.02 ± 3.36	54.66	61.37	2	173.28 ± 102.75	70.53	276.03	2	48.71 ± 4.74	43.97	53.44
8	2	407.54 ± 37.05	370.49	444.59	2	691.81 ± 114.76	577.05	806.56	2	278.69 ± 116.07	162.62	394.75
9	2	447.94 ± 61.38	386.56	509.31	2	190.23 ± 3.36	186.87	193.59	2	58.17 ± 1.38	56.79	59.54
10	3	358.52 ± 6.84	348.44	371.56	3	421.04 ± 5.83	409.78	429.33	-	-		
11	13	563.97 ± 28.90	432.89	712.00	13	584.90 ± 29.63	434.67	733.33	13	512.82 ± 30.84	375.11	698.67

Table 3. Paired-Sample t-test (taking the 2-pairs of data for each pond, selected randomly).

Differences between Leaf Groups	N	Leaf Groups	Mean ± S.E.	Paired-Sample Correlation	Paired-Sample Sig.	t-test for Equality of Means	
						t-value	p-value
(A) - (T)	11	A T	351.922 ± 70.07 187.362 ± 56.49	0.763	0.006	1.136	0.284
(T) - (B)	10	T B	294.50 ± 62.12 161.71 ± 50.40	0.767	0.010	3.322	< 0.000
(A) - (B)	10	A B	345.01 ± 77.09 161.71 ± 50.40	0.897	< 0.000	4.708	< 0.000

concentration of leaf groups A and T was found across the study ponds (Table 3). A comparison of peroxidase level of leaf-groups T and A of *A. philoxeroides* within pollution categories Low and High (Table 5) also showed no significant difference between the leaf-groups. This indicates the suitability of both A and T for estimating the peroxidase concentration in *A. philoxeroides*, T when sufficient plant tips are available at the site, otherwise opting for A.

Thus, allowing more effective and consistent sampling of young leaves of plants and minimizing the practical problem of unavailability of sufficient quantity of fresh young plant material required for the enzyme assay.

Environmental stress and peroxidase variation

Changes in levels of peroxidase were studied in natural populations of *A. philoxeroides*, in an attempt to throw some light on its ability for stress-adaptation under differing infestation as well as pollution levels.

Stress due to "A. philoxeroides infestation"

Intraspecific competition is reported to be a dominant factor for some invasive species (Blank 2010; Mangla et al. 2011). However, intraspecific competition probably does not create any stress for *A. philoxeroides*, since being a clonal plant, it is well suited to grow in

Table 4. ANOVA of Peroxidase Conc. (µg/gm F.W.) for each of the three sample groups across ponds graded into groups according to (a) *A. philoxeroides* (A. ph.) Infestation (1=low, 2=medium, 3=high) and (b) Influx of Pollution (1=low and 2=high) grades:

Leaf Sample Group	A. ph. Infestation Grade	N	Peroxidase Concentration (µg/gm F.W.) Mean ± S.E.	F	p-value
T	1	2	50.56 ± 7.46		
	2	4	326.29 ± 51.53	3.303	0.090
	3	5	379.46 ± 89.52		
A	1	2	152.44 ± 20.85		
	2	4	382.09 ± 116.15	0.895	0.446
	3	5	407.58 ± 118.02		
B	1	2	50.15 ± 1.45		
	2	4	155.06 ± 67.77	0.757	0.504
	3	4	224.15 ± 105.23		

Leaf Sample Group	Level of Pollution	N	Peroxidase Concentration (µg/gm F.W.) Mean ± S.E.	F	p-value
T	1	6	173.92 ± 58.22	13.548	0.005 *
	2	5	452.01± 43.84		
A	1	6	209.38 ± 53.64	8.879	0.015 *
	2	5	522.97 ± 96.32		
B	1	6	71.99 ± 15.24	8.951	0.017 *
	2	4	296.29 ± 91.72		

*Significant difference at p = 0.05

Table 5. Paired t-test for comparing means of peroxidase level of leaf groups T and A of *A. philoxeroides* within pollution categories.

Level of Pollution	N	Correlation		df	Significance (paired) (2-tailed)
		r	Sig		
Low	6	0.803	0.054	5	0.362
High	5	0.234	0.705	4	0.500

dense-strands and the specific clonal network might be a factor facilitating their expansion in diverse habitats (Wang et al. 2009; Xu et al. 2010). Physiological integration facilitates coordinated growth (Holzapfel and Alpert 2003), ability to exchange substances via vascular transport of resources (Pitelka and Ashmun 1985), increases cooperation (de Kroon and Groenendael 1997) and is a major ecological advantage of clonal plants (Du et al. 2010). We, therefore, hypothesize in this case, that high levels of infestation would not create any intra-specific stress among the plants (due to competition), reflected through peroxidase levels, possibly because of their clonal vegetative growth, which allows them to spread without any hindrance.

From Table 4, it was evident that there were no significant differences in mean peroxidase concentration (µg/gm F.W.) for each of the three leaf groups across ponds graded into High, Medium and Low, on the basis of "*A. philoxeroides* infestation" thereby confirming our hypothesis that strand-density did not have any stress-related effect on *A. philoxeroides*. On the contrary, this might indicate that increased strand-density could be a beneficial factor for the survivality and propagation of *A. philoxeroides* in aquatic habitats. The ability of the plant to propagate vegetatively is probably instrumental for its spread and its high rate of growth may be one reason for its high degree of colonization (Zuo et al. 2012). Positive density dependent growth was also reported by Cain et al. (1995) in the clonal plant *Trifolium repens*.

Stress due to "Level of Pollution"

Peroxidase activity in plant tissues has been suggested as an indicator of environmental pollution stress in both air (Keller 1974, Varshney and Varshney 1985) and water (Roy et al. 1992, Nimptscha et al. 2005). Specifically in aquatic systems, increase in peroxidase activity has been used as a biochemical indicator of increased organic (Santra et al. 2003) and toxic chemical (Byl et al. 1994) pollution. In view of the above, an attempt was made to correlate levels of peroxidase with differences in the High and Low pollution grades of the study ponds.

Significant difference in the mean peroxidase concentration of *A. philoxeroides* was found in the pond groups (High and Low) categorized on the basis of "Level of Pollution", with respect to all the three leaf group samples T (p=0.005), A (p=0.015) and B (p=0.017) (Table – 4). It can be seen from Table – 2 that a general trend of higher peroxidase values (>300 µg/gm FW) in *A. philoxeroides* in groups (T) and (A) could be observed in case of ponds with higher influx of wastes (namely Pond 5, and 8 - 11), which might support the assumption that higher peroxidase concentration enhances the tolerance of *A. philoxeroides* to adapt to stress conditions. Zou et al (2012) also reported that aquatic *A. philoxeroides* showed higher amount of peroxidase, which provides a greater resistance to adverse conditions in aquatic ecosystems. Peroxidase activity in aquatic plants was considered to be a critical determinant of tolerance to water pollution caused by pulp and paper mill effluents (Roy et al. 1992), other industrial effluents (Nayek et al. 2010) and also to heavy metals (Naqvi and Rizvi 2000), like copper (Xu et al. 2011a), zinc (Yuan et al. 2009) and cadmium (Ding et al. 2007).

Conclusion

This study shows evidence in favour of two specific traits of *Alternanthera philoxeroides* that are reported to facilitate plant adaptations in different environments, namely, its ability to grow even under high strand density (through absence of stress due to infestation) and its ability to withstand a broad range of environmental conditions (through increase in peroxidase concentration in plants exposed to higher influx of pollution). The capacity of *A. philoxeroides* to grow and proliferate in different grades of pond ecosystems and its wide adaptability might be due to the high anti-oxidative protection provided by its stress enzymes. Further studies are needed to understand the mechanism of enhanced peroxidase levels under stress due to eutrophication. Collection of adequate amounts of leaf samples for enzyme assays by using the young leaves along with the fresh tips (leaf buds) makes the collection and extraction process much faster and more efficient thereby helping in better assessment of the ecological health and status of plant species.

Unfortunately, *A. philoxeroides* has not yet been granted any special invasive/pest species status at the governmental level in India, although it has been reported to be an invasive alien flora of India (Reddy et al. 2008). On the other hand, in view of its negative ecological impacts, this plant has already been identified and given a special "weed-status" in many other countries, for instance, in USA (http://www.plants.usda.gov), Tasmania (http://www.dpiw.tas.gov.au), New Zealand (http://www.waikatoregion.govt.nz) and Australia (http://www.dpi.nsw.gov.au). *A. philoxeroides* was added to the EPPO Alert List in 2007 and transferred to the List of Invasive Alien Plants in 2012 (http://www.eppo.nint) showing the increasing cause of concern for this species.

This study points to the presence of *A. philoxeroides* infestations of varying population sizes in pond ecosystems in Kolkata, India. Its ability to survive as a dominant flora in association with other plants also constitutes a serious threat to other native plants and organisms. The ability of the plant to occupy disturbed habitats, a trait of a successful invader (Cadotte et al. 2006), complemented with its ability to withstand stress reflected through its higher peroxidase levels indicates its capability to become a successful invader. This paper emphasizes the latent risk of rapid invasion of *A. philoxeroides* in aquatic ecosystems and wetlands and highlights the urgency for creating awareness, both at the public and governmental level, about its potential risk of spread and thereby initiation of proper management procedures before expensive control measures become imperative.

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