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Azolla pinnata Growth Performance in Different Water Sources

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Abstract: *Azolla pinnata* R.Br. growth performance experiments in different water sources were conducted from May until July 2011 at Aquaculture Research Station, Puchong, Malaysia. Four types of water sources (waste water, drain water, paddy field water and distilled water) each with different nutrient contents were used to grow and evaluate the growth performance of *A. pinnata.* Four water sources with different nutrient contents; waste, drain, paddy and distilled water as control were used to evaluate the growth performance of *A. pinnata*. Generally, irrespective of the types of water sources there were increased in plant biomass from the initial biomass (e.g., after the first week; lowest 25.2% in distilled water to highest 133.3% in drain water) and the corresponding daily growth rate (3.61% in distilled water to 19.04% in drain water). The increased in biomass although fluctuated with time was consistently higher in drain water compared to increased in biomass for other water sources. Of the four water sources, drain water with relatively higher nitrate concentration (0.035±0.003 mg L^{-1}) and nitrite (0.044±0.005 mg L^{-1}) and with the available phosphate (0.032±0.006 mg L^{-1}) initially provided the most favourable conditions for *Azolla* growth and propagation. Based on BVSTEP analysis (PRIMER v5), the results indicated that a combination of more than one nutrient or multiple nutrient contents explained the observed increased in biomass of *A. pinnata* grown in the different water sources.

Key words: *Azolla pinnata,* water sources, number of plants, biomass production, daily growth

INTRODUCTION

The genus *Azolla* was established by Lamarck in 1783 belongs to division Pteridophyta, under the family Azollaceae. According to Lumpkin and Plucknett (1980, 1982), *Azolla* consists of two subgenera and six species. The subgenera *Euazolla*, characterized by three megaspore floats and septate glochidia, comprises four species; *Azolla filiculoides* Lam., *A. caroliniana* Willd., *A. microphylla* Kaulf. and *A. mexicana* Presl. The subgenera *Rhizosperma*, characterized by nine megaspore floats, comprises two species; *A. pinnata* R.Br. with simple glochidia and *A. nilotica* Decne with no glochidia.

Azolla is a small, free-floating water fern commonly found on calm waters such as ditches, paddy fields, ponds and coffee fields (Lumpkin and Plucknett, 1982; Anand and Pereira, 2006; Wagner, 1997; Kannaiyan, 2002). *Azolla* is also known as Mosquito fern growing as thick mat on the water's surface and prevent mosquitoes from breeding. It is a unique plant in symbiosis with blue-green alga, *Anabaena azollae* in its leaves that fixes atmospheric nitrogen (Lumpkin and Plucknett, 1980; Anand and Pereira, 2006; Wagner, 1997). Due to this symbiotic partnership, *Azolla* multiply very fast and incorporating high nitrogen, thus making it as desirable organic fertilizer either as fresh, dried or composted (Lumpkin and Plucknett, 1980; Lumpkin, 1987; Khan, 1983; Wagner, 1997; Kannaiyan, 2002). *Azolla* has been used for several decades as green manure in rice fields (Peters and Meeks, 1989), suitable and effective biofertilizer for fruits or ornamental plants and as an alternative fish meal in the diet of *Labeo rohita* fry due to its protein content (Sheeno and Sahu, 2006).

There are several studies on *Azolla* pertaining to its botany, physiology and its roles as green manure (Lumpkin and Plucknett, 1982), in agriculture (Khan, 1983) and in rice culture improving the nitrogen-fixing systems (Watanabe, 1977; Watanabe and Liu, 1992) and responses to heavy metals pollution (Sarkar and Jana, 1985), elevated CO_2, temperature and phosphorus levels (Weiguo *et al*., 2010). In agriculture the beneficial roles of *Azolla* are many as mentioned above and one of them is as nitrogen fertilizer that can be utilized to potentially increase crop yields. Thus, the evolution of culture

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practice in increasing or enhancing plant production yield should incorporate the use of *Azolla* as fertilizer. In this respect one has to increase *Azolla* cultivation to meet the demand for supply of an alternative fertilizer as opposed to the commercial available fertilizer such as urea. *Azolla* occurs in moist environments or habitats with stagnant water such as reservoirs, swamps, drains, ditches, ponds, sewage, lakes and paddy field (Lumpkin and Plucknett, 1982). In these habitats, *Azolla* survives and expands its population but probably with varied growth performance depending on the availability and content of nutrients and minerals in the different water sources. Being aquatic plant in environments with diverse water sources and qualities it is not known how they respond to survive and continue their propagation. Therefore, this study carried out laboratory experiments on *Azolla pinnata* response variables i.e., number of plants, biomass and growth rate against the tested four water sources with varied nutrient contents.

MATERIALS AND METHODS

Azolla pinnata R.Br. growth performance experiments in the different water sources were conducted from May until July 2011 at Aquaculture Research Station, Puchong, Malaysia. Four water sources with different nutrient contents; paddy field (MAEPS, Serdang), waste water (MAEPS, Serdang), drain (MARDI, Serdang) and distilled water as control were used to evaluate the growth performance of *Azolla pinnata*. All water resources were filtered through glass fiber filters (GF6, Schleicher and Schuell) for the experiments described below and determination of nutrients, ammonia (NH_3), nitrate (NO_3), nitrite (NO_2) and phosphate (PO_4) were conducted. Approximately 0.20 g of fresh weight *Azolla pinnata* were grown in 400 mL of distilled water in 16 separate 26.8×27.9 cm polyethylene zipper plastic bags. Similar experimental setup was also performed with the replacement of distilled water with water sources from paddy field, waste and drain as mentioned above. The quantity of water added allows the *Azolla* to float at the surface of water with depth of 4.5 cm. Aeration was supplied to all the *Azolla* bags to ensure constant supply of air and maintained the temperature during the study period. Four bags from each treatment were collected and processed weekly for the counting of plants produced, to record their biomass and simultaneously the biomass data was used to calculate the increased in biomass weekly and percent daily growth rate. The water from each bag was recorded for temperature and pH (HANNA instrument), retrieved and filtered through glass fiber filters (GF6, Schleicher and Schuell). Both the water sources used at the initial of the experiments and weekly retrieved filtered water for each water sources were analysed for ammonia (NH_3), nitrate (NO_3), nitrite (NO_2) and phosphate (PO_4) analyses following the methods in Parsons *et al*. (1984).

Statistical analyses were performed using two-way ANOVA between biomass production (increased in biomass), daily growth rate and, the water sources. Post-hoc test, i.e., DMRT, $p<0.05$ (Zar, 1984) was performed when significant differences occurred between means of the response variables. The increased in biomass of *A. pinnata* and nutrient contents in each water source was compared by using Plymouth Routines in the Multivariate Ecological Research (PRIMER) statistical software package (v.5) (Clarke and Warwick, 2001). The similarity matrix of increased in biomass was classified according to Bray-Curtis similarity (Bray and Curtis, 1957) by hierarchical agglomerative clustering via complete linkage method and followed by BV-STEP procedure to verify the nutrient or nutrients in the water sources that explained the correlation between the increased in biomass of *Azolla* in the growth experiments.

RESULTS AND DISCUSSION

Number of plants and morphological observations: The number of plants at the end of each week is shown in Fig. 1a. Irrespective of water sources the number of *Azolla pinnata* plants produced increased with time. In week 2, waste water had comparatively higher number of plants (16.75±5.63) compared to other water sources. In week 3, number of plants in waste water (8.25±1.31) and distilled water (7.50±1.19) were higher compared to drain water (4.00±0.91) and paddy field water (4.25±0.48). The observations above were obtained for *Azolla* grown and propagated in the different water sources with different nutrient content (Table 1) under water ambient temperature, 27.59-29.59°C and pH, 7.19-8.89. These growth conditions were within the range with other *Azolla* studies, temperature 20-30°C and pH 6-7 (FAO, 1978; Khan, 1983; Lumpkin, 1987; Moretti and Gigliano, 1988; Weiguo *et al*., 2010).

The growth of *A. pinnata* in each treatment varied with respect to the number of plants produced, morphology and color. *Azolla* increased its population size (the number of plants) by fragmentation (similar to observations of Wagner, 1997; Lumpkin, 1987) within 3-4 days. The increased in number of plants did not

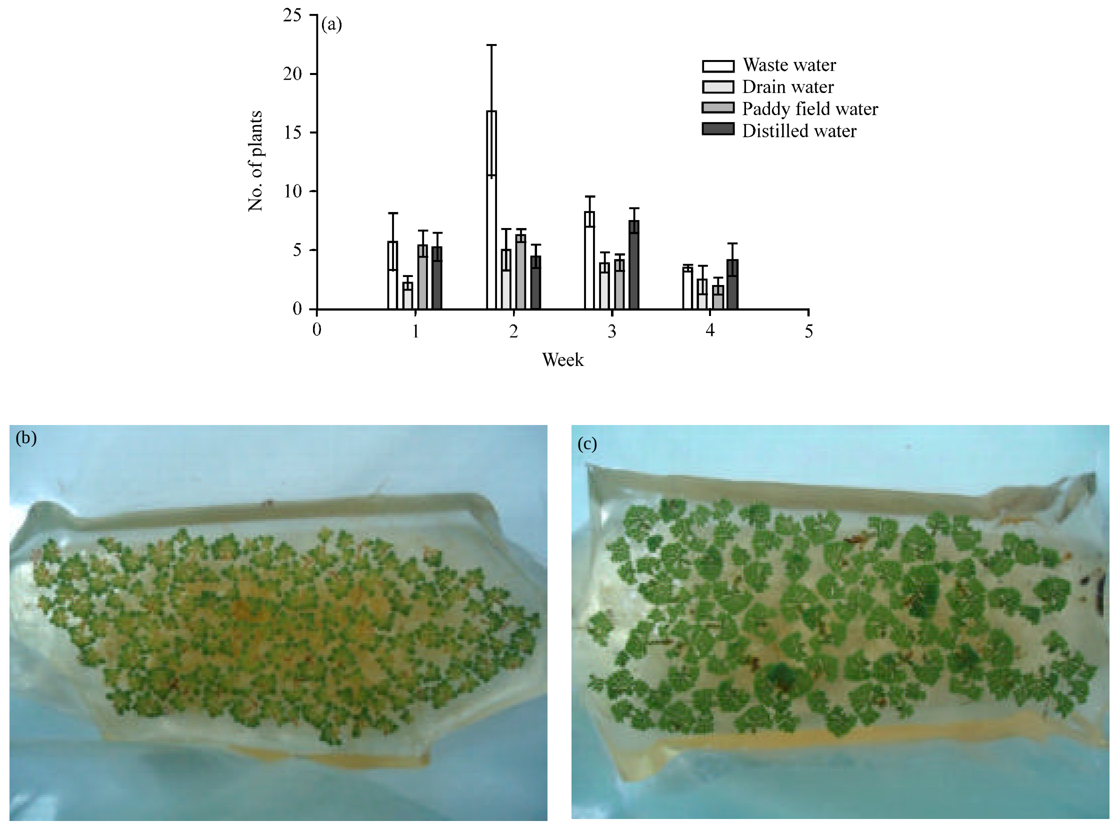

Fig. 1(a-c): *Azolla pinnata* grown in different water sources, (a) Number of plants produced per week, (b) High numbers of small-size plants in waste water and (c) Less number of plants in drain water, but their size are bigger than those in the waste water

Table 1: Nutrient in the four water sources used for *Azolla* growth experiments

	Water sources							
	Waste water		Drain water		Paddy field water		Distilled water	
Nutrient (mg L^{-1})	Mean±SE	Range	Mean±SE	Range	Mean±SE	Range	Mean±SE	Range
Ammonia (NH_4N)	0.014 ± 0.003^{a}	0.000-0.050	0.068 ± 0.012^{bc}	0.01-0.2400	0.042 ± 0.010^{ab}	0.00-0.1200	0.086 ± 0.017^{c}	0.00-0.2300
Nitrate (NO_3-N)	0.007 ± 0.002^{a}	0.000-0.037	0.035 ± 0.003^{b}	0.013-0.065	0.011 ± 0.003^{a}	0.001-0.045	0.012 ± 0.002^{a}	0.001-0.033
Nitrite (NO_2N)	0.007 ± 0.001^{a}	0.000-0.015	0.044 ± 0.005^{b}	0.013-0.091	0.010 ± 0.003^{a}	0.000-0.047	0.008 ± 0.002^{ab}	0.000-0.032
Phosphate (PO_4^{-3})	0.027 ± 0.005^{a}	0.000-0.103	0.032 ± 0.006^{a}	0.000-0.078	0.039 ± 0.009^{a}	0.005-0.145	0.023 ± 0.005^{a}	0.000-0.076

Means in the row with same alphabet are not significant different (ANOVA, DMRT, $p>0.05$)

necessarily caused high increased in biomass, since it is influenced by the plant's size as demonstrated in Fig. 1b-c. It was also observed when less fragmentation occurred, the plants were bigger in size and formed thick mat on water's surface and the resultant biomass produced was higher. Generally *Azolla* frond became yellow in color during the 15-17 days due to plant's maturation and completion of its life cycle. Plants continued to shrink and became fragile when approaching 28 days (week 4). Observations by various researchers (Watanabe, 1977; Lumpkin and Plucknett, 1980; 1982; Lumpkin, 1987; Watanabe and Liu, 1992) showed *Azolla* plants became smaller in size, less vigorous, changed color from green to pink or red and low in nitrogen content due to deficiency in phosphorus.

Biomass and growth rate: Generally, irrespective of the types of water sources there were increased in plant biomass in the first week from the initial (lowest 25.2% in distilled water to highest 133.3% in drain water, Table 2) and the corresponding daily growth rate (3.61% in

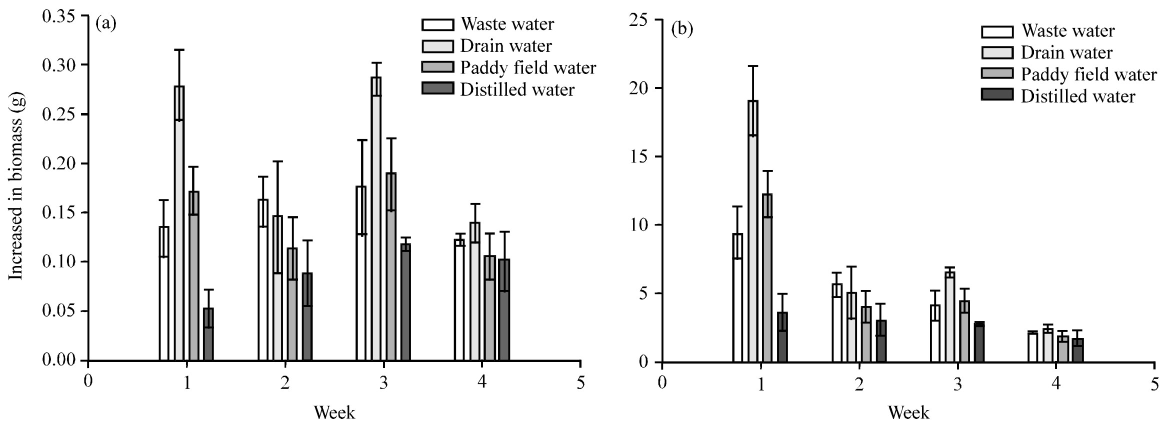

Fig. 2(a-b): (a) Increased in biomass and (b) Corresponding daily growth rate of *A. pinnata* grown in different water sources

Table 2: Increased in biomass (g), range in increased in biomass and percent increased in biomass of *A. pinnata* grown in different water sources

	Increased in biomass (g)			
Water source	Week 1	Week 2	Week 3	Week 4
Waste water				
Mean±S.E	0.135±0.028^{ab,x}	0.161±0.025^{a,x}	0.176±0.048^{a,x}	0.122±0.007^{a,x}
Range	0.060-0.200	0.090-0.200	0.080-0.300	0.110-0.140
Increase (%)	65.6±13.7	78.5±12.2	85.9±23.4	59.6±3.2
Drain water				
Mean±S.E	0.279±0.038^{c,y}	0.147±0.056^{a,x}	0.285±0.017^{b,y}	0.140±0.020^{a,}
^{x}Range	0.200-0.370	0.020-0.270	0.260-0.340	0.100-0.180
Increase (%)	133.3±18.2	70.0±26.7	136.3±8.3	66.6±9.4
Paddy field water				
Mean±S.E	0.172±0.024^{b,x}	0.114±0.032^{a,x}	0.190±0.037^{b,x}	0.105±0.024^{a,x}
Range	0.110-0.230	0.050-0.190	0.120-0.290	0.050-0.160
Increase (%)	85.4±11.9	56.6±16.1	94.1±18.4	52.3±11.8
Distilled water				
Mean±S.E	0.052±0.019^{a,x}	0.089±0.034^{a,x}	0.118±0.007^{a,x}	0.101±0.03^{a,x}
Range	0.010-0.100	0.020-0.170	0.100-0.130	0.040-0.170
Increase (%)	25.2±9.4	42.9±16.4	57.0±3.3	48.8±15.1

Means in the column with the same alphabet a,b,c and means in the row with the same alphabet x,y are not significant different (ANOVA, DMRT, p>0.05)

Table 3: Daily growth rate (%) and range of *A. pinnata* grown in different water sources

	Daily growth rate (%)			
Water source	Week 1	Week 2	Week 3	Week 4
Waste water				
Mean±S.E	9.37±1.95^{ab,y}	5.61±0.87^{a,x}	4.09±1.12^{a,x}	2.13±0.11^{a,x}
Range	4.460-13.590	3.070-7.000	1.860-6.990	1.860-2.390
Drain water				
Mean±S.E	19.04±2.60^{c,y}	5.00±1.91^{a,x}	6.49±0.40^{c,x}	2.38±0.34^{a,x}
Range	13.720-25.390	0.650-9.210	5.960-7.670	1.660-3.070
Paddy field water				
Mean±S.E	12.21±1.71^{b,y}	4.05±1.15^{a,x}	4.48±0.88^{bc,x}	1.87±0.42^{a,x}
Range	8.090-16.390	1.920-6.780	2.910-6.860	0.080-2.820
Distilled water				
Mean±S.E	3.61±1.05^{a,x}	3.07±0.91^{a,x}	2.72±0.12^{a,x}	1.74±0.42^{a,x}
Range	0.490-6.930	0.760-5.890	2.270-3.010	0.730-2.930

Means in the column with the same alphabet a,b,c and means in the row with the same alphabet x,y are not significant different (ANOVA, DMRT, p>0.05)

distilled water to 19.04% in drain water, Table 3). The increased in biomass although fluctuated with time was consistently higher in drain water compared to increased in biomass for other water sources. However, in week 4, the increased in biomass decreased for each treatment (Table 2, Fig. 2a).

Table 4: The drivers, i.e., nutrient combination related to the observed increased in biomass in *Azolla pinnata* throughout the growth experiments as produced by BV-STEP analysis

Biomass/water sources	No. of nutrient	Spearman rank correlation (p)	Best nutrient combination
Biomass			
Waste water	3	0.543	Nitrite, phosphate, nitrate
Drain water	3	0.543	Nitrite, phosphate, nitrate
Paddy field water	2	0.371	Ammonia, nitrate
Distilled water	3	0.829	Ammonia, nitrate, phosphate

Daily growth rate of *Azolla pinnata* in all water sources was inversely proportional with time (Fig. 2b). This is the characteristic pattern for plants with short life cycle such as *Azolla*, attaining maximum growth after the first week and thereafter growth tend to decline as the plant have reached maturity and eventually died. As *A. pinnata* could fixed nitrogen from the atmosphere, plants death was not due to lack of nutrient concentration in the water sources, rather it is the attribute to the life span of the plant. In almost all cases, the observed daily growth rate of *A. pinnata* in drain water was relatively higher when compared with other water sources (Fig. 2b). *Azolla pinnata* grew and propagated well in drain water throughout the study period as this Means in the column with the same alphabet (a,b,c) and means in the row with the same alphabet (x,y) are not significantly different (ANOVA, DMRT, $p>0.05$) water source contained high concentration of nitrate (0.035 ± 0.003 mg L^{-1}) and nitrite (0.044 ± 0.005 mg L^{-1}) compared to waste, paddy field and distilled water (Table 1, $p<0.05$). In contrast phosphate content was not significantly different (ranging from 0.023 ± 0.005 to 0.039 ± 0.008 mg L^{-1}) in all water sources while ammonia content was significantly higher in waste water (0.014 ± 0.003 mg L^{-1}) and distilled water (0.086 ± 0.017 mg L^{-1}) (Table 1, $p<0.05$).

Plants such as *A. pinnata* may not selectively absorbed one nutrient at a time, rather the uptake may involve a combination of absorbable nutrient available in the water sources. To evaluate this a multivariate non-parametric procedure BV-STEP, implemented in the software PRIMER-E (Clarke and Warwick, 2001), was applied for increased in biomass in *Azolla* in the four growth experiments to assess related drivers, i.e., nutrients (nitrate, nitrite, ammonia and phosphate content) of water sources. The results indicated a combination of more than one nutrient or multiple nutrients (Table 4) in the water sources that explained the observed increased in biomass of *A. pinnata*. The correlation between the variables is described by the Spearman rank correlation (ρ) which ranges from +1 (perfect correlation), through 0 (no correlation), to -1 (perfect negative correlation). The ρ values up to 0.33 are considered weak relationships, between 0.34 to 0.66 medium strength relationships and over 0.67 as strong relationships. For *A. pinnata* grown in drain and waste water, the combination for nutrient contents responsible for the increased in biomass were nitrite, phosphate and nitrate with ρ 0.543. In the case of *A. pinnata* grown in paddy field water, ammonia and nitrate was a combination responsible for the biomass and daily growth rate with ρ 0.371. Ammonia, nitrate and phosphate with ρ 0.829 was the best combination responsible for biomass of *A. pinnata* grown in distilled water. A graphical representation given in Fig. 3 showed the potential drivers, i.e., nutrient combination (Table 4) as produced through analysis by BV-STEP plotted with time and the corresponding increased in biomass of *A. pinnata* with time in different water sources. The presence of nutrients and the concentration in the water sources (Fig. 3 as shown by line graph) although fluctuated with time provided the necessity for growth and propagation of *A. pinnata*. According to Ito and Watanabe (1983), the presence of ammonium or nitrate indicated that *Azolla* continued to fix nitrogen even at a lower rate. *Azolla pinnata* is a floating aquatic plant, absorbed nutrients via its roots andfronds similar to *Landoltia punctata,* which absorbed nitrogen via similar structures (Fang *et al*., 2007). When both sources of N are available, plants prefer to absorb nitrogen in NH_4^+ rather than in NO_3^-. Accordingly, the presence of NH_4^+ or NO_3^- in water sources enable *Azolla* in the uptake of N by exceeding the corresponding decrease in N_2 fixation, resulting in increase of N concentration (Sah *et al*., 1989).

The growth performance for *A. pinnata* can vary with the types of water sources in term of number of plants produced, increased in biomass yield and growth rates which may be attributed to a combination of factors involved (e.g., fragmentation process, nutrient availability and content and perhaps mineral availability and content e.g., potassium). Based on this present study, *A. pinnata* grown in various water sources, under the ambient growing conditions, higher number of plants produced (depending on size of plants) may not necessarily produced the corresponding increase in biomass, e.g., comparing *Azolla* growth in drain water and waste water after three weeks; although the number of plants (4.00 ± 0.91 plants, Fig. 1a) in drain water was less than those in waste water (the number of plants, 8.25 ± 1.31, Fig. 1a) but the corresponding increased in biomass (0.285 ± 0.017 g $week^{-1}$ or 136.3%, Table 2) compared with waste water, the increased in biomass (0.176±0.048 g week^{-1} or 85.9%, Table 2) was high. Plants were bigger in size and hence more biomass as compared to small plants observed in waste water. Better growth performance occurred in water source with high readily available nitrite, nitrate and with the presence of phosphate as in the case drain water.

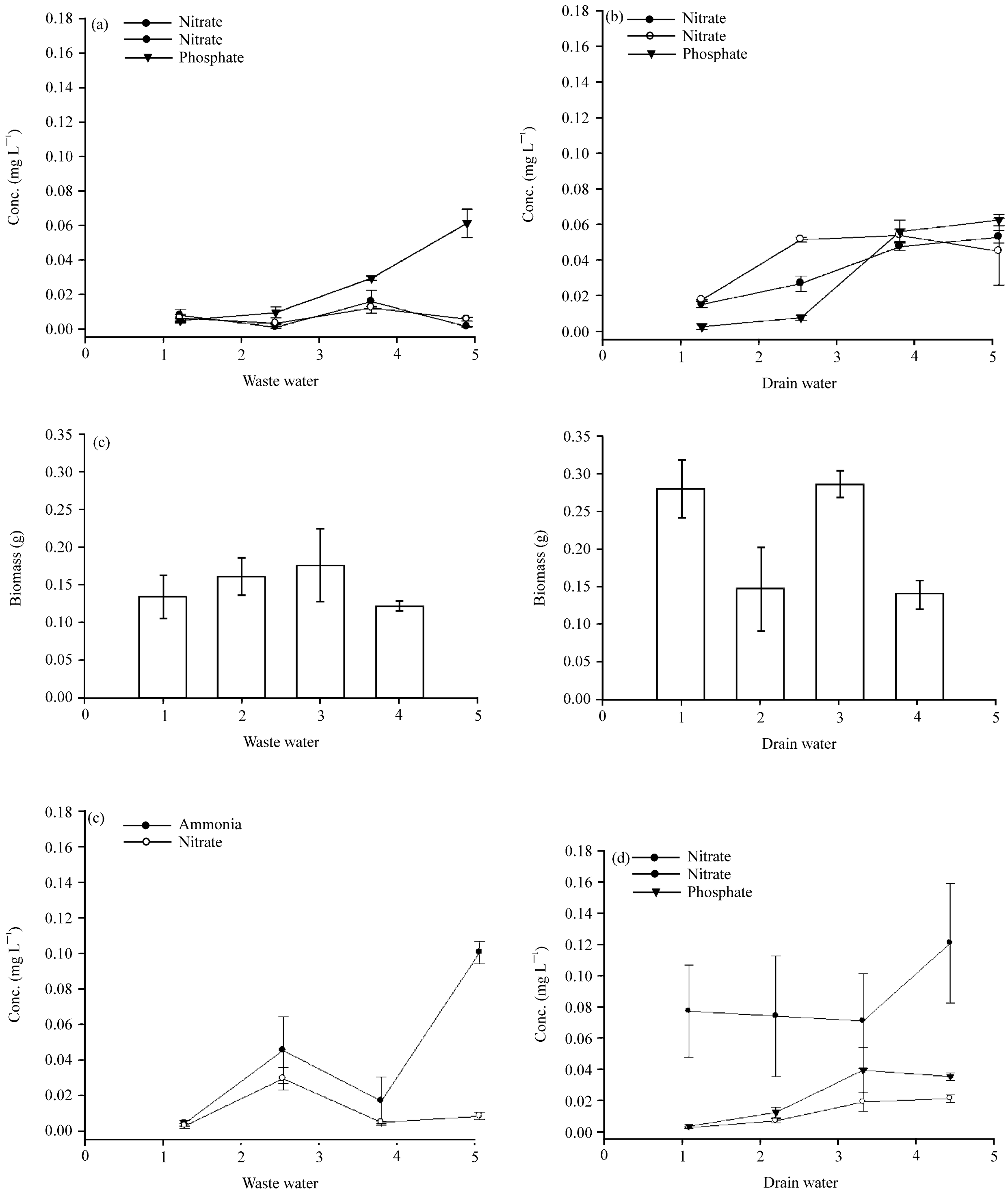

Fig. 3(a-d): Continued

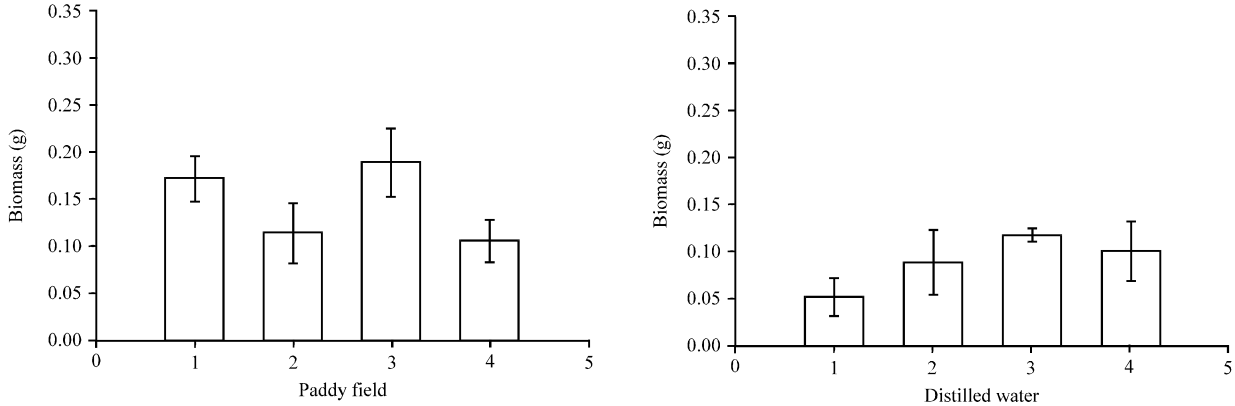

Fig. 3(a-d): The drivers, i.e., nutrient combination (Table 4 as determined by BV-STEP analysis and as indicated by line graphs) related to the increased in biomass of *A. pinnata* in different water sources. Nitrite, phosphate and nitrate was the combination responsible for the increased in biomass of *A. pinnata* grown in (a) Waste water and (b) Drain water, ammonia and nitrate was the combination responsible for the biomass in (c) Paddy field and ammonia, nitrate and phosphate was the combination responsible for the biomass in (d) Distilled water

CONCLUSION

Generally, the growth performance and general morphology irrespective of the types of water sources showed variable increased in plants' number and biomass from the initial biomass and the corresponding daily growth rate. A specific combination of nutrient contents (nitrite, phosphate, nitrate or ammonia) in the water resources were responsible for the increased in plant's number and biomass.

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