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ECOLOGY

Performance of *Azolla caroliniana* Willd. and *Salvinia auriculata* Aubl. on fish farming effluent

Performance de *Azolla caroliniana* Willd. e *Salvinia auriculata* Aubl. em efluente de piscicultura

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ABSTRACT

The increasing release of untreated fish farming effluents into water courses that flow to the Pantanal wetlands in Mato Grosso (Brazil) may drive this ecosystem to eutrophication. Therefore, the growth of *Azolla caroliniana* Willd. and *Salvinia auriculata* Aubl. in fish farming effluent and their effect on its quality were evaluated for 48 days in a greenhouse. The results were compared to those obtained in a nutrient rich solution (Hoagland ½ medium). *Azolla caroliniana* showed lower relative growth rate in fish farming effluent (0.020 d^{-1}) than in Hoagland ½ medium (0.029 d^{-1}). However, *S. auriculata* grew slightly better in fish farming effluent (0.030 d^{-1}) than in Hoagland ½ medium (0.025 d^{-1}). The species apparently contributed to reduce nitrate and phosphate concentration in Hoagland ½ medium. However, in fish farming effluent, only electrical conductivity and pH were reduced by plants compared to the control without plants. Thus, *A. caroliniana* and *S. auriculata* show low potential for improving effluent quality.

Keywords: fish farming effluent treatment, nutrients, Mato Grosso, native macrophyte species, Pantanal wetlands.

RESUMO

A crescente liberação de efluentes de piscicultura sem tratamento nos cursos d'água que alimentam as áreas inundáveis do Pantanal Mato-grossense (Brasil) pode conduzir à eutrofização desse ecossistema. Assim, o crescimento de *Azolla caroliniana* Willd. e *Salvinia auriculata* Aubl. e seu potencial para melhoramento da qualidade

de efluente de piscicultura foi avaliada por 48 dias em casa de vegetação. Os resultados foram comparados aos obtidos em uma solução nutritiva (meio Hoagland ½). *Azolla caroliniana* apresentou menor taxa de crescimento relativo em efluente de piscicultura (0.020 d^{-1}) do que em meio Hoagland ½ (0.029 d^{-1}). Entretanto, *S. auriculata* apresentou um crescimento ligeiramente mais rápido em efluente de piscicultura (0.030 d^{-1}) do que em meio Hoagland ½ (0.025 d^{-1}). As duas espécies aparentemente contribuíram para redução das concentrações de nitrato e fosfato no meio Hoagland ½. No entanto, em efluente de piscicultura, somente a condutividade elétrica e o pH tiveram os valores reduzidos pelas plantas em comparação com o controle sem plantas. Assim, *A. caroliniana* e *S. auriculata* apresentam baixo potencial para melhorar a qualidade do efluente de piscicultura.

Palavras-chave: tratamento de efluente de piscicultura, nutrientes, Mato Grosso, espécies nativas de macrófitas, Pantanal.

1. Introduction

Fish farming is an ever-increasing activity in the surroundings of the Pantanal wetlands of Mato Grosso (Brazil). While fishes are cultivated (and at the harvesting), effluents containing nutrients, toxins, proteins and particulate material are released into water courses that flow into the Pantanal wetlands. As fish farming effluent can drive aquatic ecosystems to eutrophication (Henry-Silva and Camargo, 2008a), it is necessary to treat them using low cost practices compatible with the low economic conditions of many fish farmers in the Pantanal region. The use of aquatic macrophytes for treatment of domestic effluents has been encouraged due to its low cost and efficiency for nutrient removal (Vermaat and Hanif, 1998; Costa et al., 1999; Sooknah and Wilkie, 2004; Olguín et al., 2007). Although the utilization of aquatic macrophytes for treatment of aquaculture effluents has already been investigated (Redding et al., 1997; Forni et al., 2001), research on this practice is still incipient in Brazil (Sipaúba-Tavares et al., 2002; Henry-Silva and Camargo, 2006a, 2008b).

Azolla and *Salvinia* are genera of free-floating macrophytes that have shown high potential for treatment of domestic, human and pig wastes (Sarkar, 1986; Kitoh et al., 1993; Vermaat and Hanif, 1998; Costa et al., 1999; Olguín et al., 2007). However, their potential for treatment of fish farming effluents has not yet been well investigated (Redding et al., 1997; Forni et al., 2001; Henry-Silva and Camargo, 2006a). Some attention has been paid to *Azolla* due to its potential use as food for fish and biofertilizer (Wagner, 1997). *Azolla* species maintain a symbiotic relationship with the cyanobacteria *Anabaena azollae*, which fix atmospheric nitrogen for the plant in exchange for carbohydrates (Arora and Singh, 2003); hence *Azolla* plants are rich in nitrogen and protein. Nevertheless, other macrophyte genera (including *Salvinia*) used for treatment of aquaculture effluents also showed potential as food for fish and livestock (Henry-Silva and Camargo, 2002, 2006b; Henry-Silva et al., 2006).

Azolla caroliniana Willd. (Azollaceae) and *Salvinia auriculata* Aubl. (Salvinaceae) are abundant aquatic macrophyte species in the Pantanal wetlands and show high propagation capacity. These species could be used for treatment of fish farming effluent in the Pantanal region and their biomass could be utilized as biofertilizer or food for livestock. Therefore, we experimentally analyzed the potential of *A. caroliniana* and *S. auriculata* for growth and for improving the quality of a fish farming effluent of the Pantanal region. Also, we compared the results to those obtained in a rich nutrient solution to determine how the performance of the two species would be under high nutrient availability.

2. Material and Methods

Salvinia auriculata and *A. caroliniana* were collected in dams (16° 06'-21' S and 56° 27'-34' W) of the Poconé region in the Pantanal wetlands, Brazil. Before starting the experiment, the plants were kept in Hoagland ½ medium (Passos, 1996) for 15 days under conditions of temperature and luminosity similar to those maintained throughout the experimental period (see below).

The experiment was carried out in a greenhouse at Universidade Federal de Mato Grosso, in Cuiabá, Brazil. The plants were grown for 48 days in white plastic baths (39.2 × 25.5 × 8 cm) containing 5 L of the culture medium (≅ 5 cm deep). Experimental design consisted of three random blocks, where each block was a replication. We cultivated *A. caroliniana* and *S. auriculata* in a fish farming effluent collected from outflows of ponds located at Estação Experimental de Piscicultura of the Empresa Mato-Grossense de Pesquisa e Extensão Rural, municipality of Nossa Senhora do Livramento (15° 46' 00" S and 56° 19' 03" W), Brazil. We left three baths filled with effluent without plants (control) to evaluate changes on the physical and chemical characteristics of the water. The plants were also grown in a Hoagland rich nutrient solution (Passos, 1996) modified and half diluted (Hoagland ½ medium). Modifications made to the Hoagland ½ medium were substitution of KH_2PO_4 by K_2HPO_4, use of $CaSO_4.2H_2O$ as a source of Ca^{2+} and use of KNO_3 as a source of NO_3^-; NO_3^- concentration was increased five times and K^+ was doubled. Physical and chemical characteristics of the Hoagland ½ medium and of the fish farming effluent were measured at zero, 30 and 48 days of culture. Water temperature was measured with a WTW thermometer and electrical conductivity with a WTW LF conductivimeter. A Lutron pH-206 pH meter was used to

measure pH. Nitrate, nitrite, ammonia and phosphate were determined through methods of cadmium column, colorimetry, phenate and ascorbic acid, respectively (APHA, 1998). Total carbon was measured through a chromatographic method (Multi N/C 3000, Analytik Jena).

Water loss by evapotranspiration was replaced on a weekly basis using deinonized water. During the experimental period, air temperature was 27.4 ± 2.1 °C (mean ± standard deviation) (23.8-30 °C, min.-max.) and photosynthetic active radiation (PAR) was 3.7 ± 1.5 $MJ.m^{-2}.d^{-1}$ (1.5-5.4 $MJ.m^{-2}.d^{-1}$). We considered PAR as 50 % of the total solar radiation since PAR corresponds to the wavelengths between 400 to 700 nm, which contribute to approximately 42 to 50% of the total radiation. We calculated the total solar radiation that reaches the top of the atmosphere using the number of hours of solar radiation per day measured at a nearby meteorological station (~10 km of distance) at a latitudinal position of 15.55° S through equations described in Vianello and Alves (1991). Flux of photosynthetic active radiation, measured with a LI-COR - 6400 on a sunny day, was 39.7 ± 36.9 $\mu E.m^{-2}.s^{-1}$ (10-123 $\mu E.m^{-2}.s^{-1}$).

Initial amount of plants inoculated in each bath was 12.5 g of fresh biomass. Fresh plant biomass was estimated at zero, 20, 35 and 48 days of culture. Before weighing, the plants were rinsed with deionized water and blotted on tissue paper for five minutes to remove excess of water (Vermaat and Hanif, 1998; Forni et al., 2001; Henry-Silva and Camargo, 2008b). At the end of the experiment all plants were harvested and dried out. Drying was done at 40 °C for 72 hours in a vented oven and then in a muffle furnace at 105 °C for three hours.

Regressions to convert fresh biomass (FB) into dry biomass (DB) were obtained with samples harvested before starting the experiment and with all plants harvested in the last weighting. A linear regression was applied to *A. caroliniana* (DB = 0.06939 + 0.03635*FB, GL = 1; 36, r^2 = 0.94; DB and FB in grams) and another to *S. auriculata* (DB = 0.07408 + 0.02492*FB, GL = 1; 39, r^2 = 0.94).

We fitted an exponential model, $DB = DB_0 \times e^{rt}$, and a logistic model, $DB = K \times 1 - e^{rt}$ to growth of each species, where DB is dry biomass at time t, DB_0 is initial dry biomass, K is the asymptotic value of dry biomass and r is the intrinsic rate of increase (or relative growth rate - RGR). Model adjustment was evaluated based on the probability associated to the estimated parameters (DB_0, K and r) and on the amount of variance explained. Doubling time was estimated as ln(2)/r.

A two-way repeated-measures ANOVA was used to determine the effects of the culture media (Hoagland ½ medium and fish farming effluent) in different periods (20, 35 and 48 days) on dry biomass of *A. caroliniana* and *S. auriculata*. In this analysis we did not use plant dry biomass at zero days since there was no experimental effect on the plants yet. Repeated-measures ANOVA was also applied to test the effect of species (and control) on all water physical and chemical variables in different periods (zero, 30 and 48 days) in fish farming effluent and Hoagland ½ medium separately. Before running all analyses we evaluated the assumption of normality of the data using the Kolmogorov-Smirnov one sample test, and all of them were normal. We also tested for block effect and as there was none we analyzed the data as a completely randomized design. Analyses were run in Systat 10 (Wilkinson, 2000) and R 2.8.1 (R Development Core Team, 2008).

3. Results

Although the exponential model showed low adjustment to data of *A. caroliniana* collected up to 48 days in Hoagland ½ medium, this model fitted well to data collected up to 35 days (Figure 1a) when plants were not under high negative influence of density. The exponential model provided the best fit for growth of *A. caroliniana* cultivated in fish farming effluent (Figure 1b) and for *S. auriculata* cultivated in Hoagland ½ medium (Figure 1c). It was not possible to estimate the parameters of the logistic model for *S. auriculata* cultivated in fish farming effluent because the maximum number of interactions was always exceeded; hence we used only the exponential model in this case (Figure 1d). The logistic models did not provide significant relative growth rates; hence we used those estimated from the exponential models to evaluate species growth. We used the relative growth rates estimated with data gathered up to 35 days for *A. caroliniana* cultivated in Hoagland ½ medium due to the best fit of the model to this data. *Azolla caroliniana* cultivated in fish farming effluent showed lower relative growth rate (0.020 d^{-1}) and longer doubling time (34 days) than in Hoagland ½ medium (0.029 d^{-1} and 24 days). However, *S. auriculata* grew slightly better in effluent (0.030 d^{-1} and 23 days) than in Hoagland ½ medium (0.025 d^{-1} and 28 days).

Results of repeated-measures ANOVA indicated that species growth was significantly dependent on culture medium (species × medium interaction, $F_{(1, 8)}$ = 6.4, p = 0.04). Time (days) also influenced both the growth of the species (days × species, $F_{(2, 16)}$ = 10.5, p = 0.001) and their response to each culture medium (days × medium, $F_{(2, 16)}$ = 7.1, p = 0.006). Dry biomass of *A. caroliniana* increased faster in Hoagland ½ medium (Figure 1a) than in fish farming effluent (Figure 1b). At 35 days of culture, this species reached 18.4 ± 2.3 g $DB.m^{-2}$ in Hoagland ½ medium and only 12.5 ± 3.3 g $DB.m^{-2}$ in fish farming effluent. However, after this period the plants started dying and at the end of the experiment, the biomass reached in Hoagland ½ medium (11.4 ± 0.3 g $DB.m^{-2}$) was slightly lower than that in fish farming effluent (13.1 ± 3.9 g $DB.m^{-2}$). *Salvinia auriculata* did not show high difference between the amount of biomass in Hoagland ½ medium (8.8 ± 1.1 g $DB.m^{-2}$) and in fish farming effluent (7.5 ±

1.6 g DB.m^{-2}) at 35 days of culture (Figure 1c, d). Nevertheless, at the end of the experiment the biomass of *S. auriculata* in fish farming effluent (13.3 ± 5.2 g DB.m^{-2}) was slightly higher than that in Hoagland ½ medium (11 ± 2.2 g DB.m^{-2}).

Results of repeated measures ANOVA for physical and chemical variables in Hoagland ½ medium and fish farming effluent are shown in Figure 2 and 3, respectively. Water temperature increased significantly (~22-23.5 °C) in the Hoagland ½ medium (Figure 2a) and in fish farming effluent with or without plants (Figure 3a). In Hoagland ½ medium, the electrical conductivity varied little (staying around 2.5 µS.cm^{-1}) and did not change through time (Figure 2b). However, there was a higher increase of pH in water containing *S. auriculata* (6-7.8) than *A. caroliniana* (6-6.8) (Figure 2c). Nitrate decreased (~80-60 mg.L^{-1}) in water with the two species (Figure 2d) while nitrite increased more in water with *S. auriculata* (0.03-0.27 mg.L^{-1}) than with *A. caroliniana* (0.03-0.13 mg.L^{-1}) (Figure 2e). Ammonia increased in water with *A. caroliniana* (0.15-1.43 mg.L^{-1}) but decreased in water with *S. auriculata* (0.14-0.09 mg.L^{-1}) (Figure 2f). Phosphate unexpectedly decreased more in water with *S. auriculata* (4.9-1.8 mg.L^{-1}) than with *A. caroliniana* (4.8-2.8 mg.L^{-1}) (Figure 2g). Total carbon thoroughly increased (~10-30 mg.L^{-1}) up to 30 days in water with the two species (Figure 2h), but decreased afterwards, reaching 8.7 and 17.8 mg.L^{-1} for *A. caroliniana* and *S. auriculata* (respectively).

The electrical conductivity decreased more in fish farming effluent containing plants (~194-100 µS.cm^{-1}) than without plants (193-144 µS.cm^{-1}) (Figure 3b). Reduction of pH was obtained in effluent with plants (~7.9-7.5) while an increase occurred in effluent without plants (8-8.6) (Figure 3c). Nitrate and nitrite were quite variable and did not show significant trends, though nitrate apparently increased and nitrite decreased (Figure 3d-e). However, ammonia strongly decreased in effluent with *A. caroliniana* (6.4-0.08 mg.L^{-1}), in the control without plants (6.8-0.03 mg.L^{-1}) and in effluent with *S. auriculata* (6.5-0.001 mg.L^{-1}) (Figure 3f). Phosphate decreased up to 30 days, but increased afterwards though not significantly (Figure 3g). Total carbon had a significant increase of concentration (~15 to 24 mg.L^{-1}) up to 30 days, but had a decrease up to 48 days to 18 mg.L^{-1} in the control and 11 and 16 mg.L^{-1} in effluent with *A. caroliniana* and *S. auriculata*, respectively (Figure 3h).

4. Discussion

Growth of *A. caroliniana* was low since there are reports of relative growth rates over 0.1 d^{-1} for this species in nutrient rich solution (Maejima et al., 2001; Arora and Singh, 2003; Adalberto et al., 2004) and rates varying from 0.017 to 0.045 d^{-1} for *A. filliculoides* in fish farming effluent (Forni et al., 2001). *Salvinia auriculata* cultivated in Hoagland ½ medium showed relative growth rate higher than *S. herzogii* (0.016 d^{-1}) (Panigatti and Maine, 2003) but lower than *S. molesta* (0.045-0.17 d^{-1}) and *S. minima* (0.088-0.175 d^{-1}) in nutrient rich solution (Mitchell and Tur, 1975; Sale et al., 1985, Olguín et al., 2007). In fish farming effluent, *S. auriculata* grew almost identically to *S. molesta* in fish farming effluent of low (0.029 d^{-1}) and high (0.031 d^{-1}) nutrient concentration (Henry-Silva et al., 2008). However, it grew slower than *S. minima* (0.09-0.1 d^{-1}) in pig waste anaerobic effluent (Olguín et al., 2007). Low light availability could have been a constraining factor for growth in nutrient rich solution as the studies that showed higher growth rates for *A. caroliniana* and *S. auriculata* often reported values of light intensity higher than in this study. Additionally, there is evidence that these species are able to respond in growth and nutrient uptake to an increase in light availability (Sale et al., 1985; Sarkar, 1986; Carvalho and Lopes, 1994; Petrucio and Esteves, 2000a).

Azolla caroliniana grew slower in fish farming effluent than in Hoagland ½ medium probably due to low nutrient availability. Phosphorus concentration in the fish farming effluent (0.05 ± 0.01 mg.L^{-1}) was below the minimal nutritional requirements of *A. caroliniana* (~ 3 mg.L^{-1}) (Adalberto et al., 2004). Nitrate concentration was also low in fish farming effluent. Although the association between *Azolla* and the cyanobacteria *A. azollae* would be able to supply all nitrogen necessary for the plant, nitrate supplementation can improve growth of *Azolla* even in saline conditions (Reddy et al., 1989; Rai and Rai, 2003). Nitrate concentration of 35 to 70 mg.L^{-1} is considered adequate for *A. pinnata* and *A. caroliniana* (Singh et al., 1992; Pabby et al., 2001; Rai and Rai, 2003).

Ammonia nitrogen could also have reduced the growth of *Azolla* (Kitoth et al., 1993; Maejima et al., 2001). In the water, ammonia nitrogen is present as ion ammonium (NH_4^+) and ammonia (NH_3). The former is dominant in neutral to acid pH while the latter, which is more toxic, is predominant at alkaline pH. In fish farming effluent, at the beginning of the experiment, the ammonia concentration of 6.4 mg.L^{-1} at pH 7.9 could have contributed to reduce *A. caroliniana*'s growth. Maejima et al. (2001) showed that some species of *Azolla* (including *A. caroliniana*) had slower growth rates under a high initial ammonium concentration of 280 mg.L^{-1}. However, a lower concentration (6.2 mg.L^{-1}), similar to that found in the fish farming effluent, was enough to reduce the growth of *A. filiculoides* (Kitoh et al., 1993).

Salvinia auriculata grew a little better in fish farming effluent than in Hoagland ½ medium possibly due to its ability to use ammonia nitrogen. *Salvinia auriculata* and *S. molesta* show preference to absorb ammonia nitrogen instead of nitrate (Petrucio and Esteves, 2000b; Henry-Silva and Camargo, 2006a) and absorption of phosphate by

S. herzogii increased in the presence of ammonium regardless of nitrate (Panigatti and Maine, 2003). As ammonia nitrogen demands less energy than nitrate to be converted into amino acids and proteins, it is probable that *S. auriculata* benefited in fish farming effluent. Also, an experiment with *S. minima* in nutrient rich solution and in pig waste anaerobic effluent showed that the genus may have tolerance to high initial ammonium concentration (Olguín et al., 2007). *Salvinia minima* had its growth inhibited only under a concentration of 140 $mg.L^{-1}$ of ammonium, which is much higher than the ammonia nitrogen concentration found in the fish farming effluent (6.5 $mg.L^{-1}$).

Final biomass of the two species was slightly higher in fish farming effluent than in Hoagland ½ medium, but differences between *A. caroliniana* and *S. auriculata* were very small within each medium. The significant differences apparently were only due to the faster biomass accumulation of *A. caroliniana* at 20 and 35 days of culture in Hoagland ½ medium since *Azolla* species are often able to respond in growth to high nitrate and phosphorus availability (Wagner, 1997). Biomass loss of *A. caroliniana* after 35 days was due to mortality probably caused by overcrowding.

Salvinia auriculata did not show high differences in biomass between culture media through time probably due to physiological constraints on nitrate use in Hoagland ½ medium (Petrucio and Esteves, 2000b; Panigatti and Maine, 2003; Henry-Silva and Camargo, 2006a). Henry-Silva et al. (2008) also did not find an effect of nutrient concentration on the growth of *S. molesta*, which apparently is more influenced by density due to its fast lateral growth (Camargo and Florentino, 2000; Rubim and Camargo, 2001; Henry-Silva and Camargo, 2006a).

The increase in water temperature was related to an increase in air temperature (~23-30 °C) throughout the period of culture. The increase in pH in Hoagland ½ medium occurred possibly due to the utilization of dissolved carbon by plants for photosynthesis (Henry-Silva and Camargo, 2000, 2008b), which is corroborated by a decrease in total carbon concentration at the end of the experiment. However, the higher pH in water with *S. auriculata* than *A. caroliniana* could not be explained since the amount of total carbon remaining in the water with *A. caroliniana* was lower than in the water with *S. auriculata*.

Around 30 % of nitrate was removed from Hoagland ½ medium, which is expected for water containing *A. caroliniana* (Redding et al., 1997; Forni et al., 2001), but uncommon for *S. auriculata* due to its inefficiency in absorbing nitrate (Petrucio and Esteves, 2000b; Panigatti and Maine, 2003; Henry-Silva and Camargo, 2006a). However, nitrate diminution could have occurred in part due to absorption since *S. auriculata* did not have another type of nitrogen source and due to the transformation of nitrate to nitrite through denitrifying bacteria. As the concentration of nitrite increased in the Hoagland ½ medium, this process probably took place (Panigatti and Maine, 2003). Nonetheless, denitrification needs energy, which is derived from the oxidation of organic matter in anaerobic conditions. Macrophytes may improve conditions for denitrification in nitrate rich waters by supplying organic carbon which can be used directly by denitrifying bacteria or can stimulate denitrification indirectly by contributing to a lower redox potential (Weisner et al., 1994).

The quantity of phosphate removed from the Hoagland ½ medium containing *S. auriculata* (64%) was higher than for *A. caroliniana* (41%) probably due to a return of phosphate to water through decomposition of dead plants of *A. caroliniana* at the end of the experiment. The increase of ammonia in water containing *A. caroliniana* was probably a result of decomposition of organic material (Sipaúba-Tavares et al., 1999a) from plants that died at the end of the experiment.

Higher reduction of electrical conductivity and pH in fish farming effluent containing plants was probably due to absorption of ions and retention of organic material by plant roots (Henry-Silva and Camargo, 2000, 2008b). Removal of ions and organic material from water could prevent the decomposition process and the release of substances that could increase pH and electrical conductivity. The reduction of ammonia was around 99 %, which is almost the same value for reduction in total inorganic nitrogen (data not shown). Ammonia concentration in fish farming effluent with plants was significantly different to the control, but it was very low in magnitude. No effective reduction of ammonia was detected after treatment of fish farming effluent using a biofilter of *Eichhornia crassipes* (Sipaúba-Tavares et al., 2002). Although plants could have contributed to reduce ammonia concentration through absorption (mainly *S. auriculata* due to its preference for this ion) other processes such as sedimentation, nitrification, denitrification and volatilization possibly contributed towards ammonia decrease. Nitrate and nitrite did not show significant trends, but there was a slight increase of nitrate concentration probably due to transformation from ammonia (nitrification). Decrease of phosphate would be due to plant absorption and perhaps due to algae proliferation in the effluent without plants. However, phosphate showed a slight increase after 30 days, which possibly occurred due to decomposition of organic material and death of algae (Sipaúba-Tavares et al., 1999b).

As only electrical conductivity and pH were altered by plants compared to the control without plants, *A. caroliniana* and *S. auriculata* showed low potential for improving the quality of the effluent. Nonetheless, low availability of light, nutrients and space for growth (at the end of the experiment) could have limited the plants' performance. Therefore, an investigation on nutrient uptake should be conducted in effluents with higher concentrations of phosphate and nitrate. Also, more light should be provided and the plants should be harvested periodically (as suggested by Sipaúba-Tavares et al., 2002; Henry-Silva and Camargo, 2008b) to avoid overcrowding and to increase nutrient uptake efficiency.

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