Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

Combined use of *Azolla* and loach suppressed paddy weeds and increased organic rice yield: second season results

WEIGUO CHENG *[1], MIWA TAKEI[1], CHIZURU SATO[1], VALENSI KAUTSAR[1,2], YUKA SASAKI[1], SATORU SATO[1], KEITARO TAWARAYA[1], and HIRONORI YASUDA[1]

[1]Faculty of Agriculture, Yamagata University, 1-23 Wakaba-machi, Tsuruoka, Yamagata, 997-8555 Japan
[2]Faculty of Agriculture, Gadja Mada University, Yogyakarta 55581, Indonesia

ABSTRACT

Organic farming uses alternatives to agricultural chemicals such as synthetic fertilizers and pesticides. The primary challenge in organic rice farming is controlling weeds without using herbicides and improving rice yield without chemical fertilizers. In our previous paper entitled as combined use of *Azolla* and loach suppressed weed *Monochoria vaginalis* and increased rice yield without agrochemicals, we reported the first year rice growth season results from an in situ container experiment. The experiment was designed with 4 treatments—control (with neither *Azoll*a nor loach), *Azolla* (*Azolla* alone), loach (loach alone), and Az+Lo (combined *Azolla* and loach)—with 3 replications each. The first year results showed that combined use of *Azolla* and loach was successful in weed suppression and increase in rice yield in 2012. In this paper, we report the second year results from the continuous container experiment in 2013. *M.vaginalis* emergences were very low in second year rice growth season on all treatments. Compared first year, the rice yields decreased in second year on all treatments due to different weather condition and with or without organic soybean oil cake application between two rice growth seasons. The second year results also showed the raising loach had a stronger effect to increase tiller and panicle numbers, and spikelet number per panicle, then improve rice yields to 2.3 times than control. The *Azolla* residues left from first year have weaker effect on rice growth and yield, but increase soil organic matter accumulation at second year. The two years study indicated that combined use of *Azolla* and loach can meet two of the greatest challenges in organic rice production: providing effective weed control and improving rice nutrition without agrochemicals.

Keywords: Azolla; Loach; Organic farming; Rice yield; Soil organic matter

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops in the world and is the most important food in Asia, which accounts for 90% of the total world rice production area. According to the FAOSTAT database, during the last 53 years the world rice cultivation area increased by 41% from 115 million ha in 1961 to 165 million ha in 2013, and the world average rice yield increased by 140% from 1.87 ton ha^{-1} in 1961 to 4.49 ton ha^{-1} in 2013 (FAO, 2015). The substantial increase in world rice yield, as with other crops, has mainly been achieved by use of synthetic fertilizers and pesticides, the development of new crop varieties, and improved cultivation methods. Long-term heavy application of synthetic fertilizers and pesticides, however, negatively affects the environment, induces pesticide resistance, and increases agricultural costs (Tilman et al., 2002; Brown & Funk, 2008). The growing human population requires an ever larger food supply provided by increased crop production, and agriculture must find ways to feed a growing population demanding more meat and high-calorie diets,

Correspondence: Weiguo Cheng, Faculty of Agriculture, Yamagata University, 1-23 Wakaba-machi, Tsuruoka,Yamagata, 997-8555 Japan.
Phone/Fax Tel: +81-235-28-2824; Fax: +81-235-28-2820; E-mail: cheng@tds1.tr.yamagata-u.ac.jp

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

while also decreasing its global environmental effects (Smil & Kobayashi, 2012).

According to the Codex Alimentarius Commission, “organic agriculture is a holistic production management system that avoids use of synthetic fertilizers, pesticides and genetically modified organisms, minimizes pollution of air, soil and water, and optimizes the health and productivity of interdependent communities of plants, animals and people” (http://www.codexalimentarius.org/). Many organic farming practices were carried out in the world. Seufert et al. (2012) collected the data from 62 study sites, including 34 different crop species with 316 pair organic-to-conventional yields for studying the yield difference between organic and conventional farming. The meta-analysis results showed the organic yields are typically lower than conventional yields; but under certain conditions (e.g., good management practices, specific crop types and growing conditions) organic systems can nearly match conventional yields. However, there was no rice yield data in the sources for meta-analysis (Seufert et al., 2012), though rice is one of three kind main cereal crops (wheat, rice and maize) in the world (FAO, 2015).

Rice was cultivated in Asian countries for more than thousands years ago, there were many traditional organic rice farming practices around Asian countries (Datta et al., 2009; Xie et al., 2011; Cheng et al., 2015). For improving soil fertility of rice paddy, *Azolla*, a kind of floating aquatic ferns in tropical and temperate freshwater ecosystems was used as "green manure" in rice paddies in southern China and northern Vietnam for many centuries (Watanabe & Liu, 1992; Wagner, 1997), since *Azolla* has a symbiotic N-fixing cyanobacterium (*Anabaena azollae*) within its leaves and *Azolla* modifies the physical, chemical, and biological properties of soil and the soil-water interface in rice fields (Mandal et al., 1999; De Macale & Vlek, 2004; Cheng et al., 2010). *Azolla* has been shown to suppress aquatic weeds in flooded rice fields (Janiya & Moody, 1984; Wagner, 1997; Mandal et al., 1999; Sahoo & Datta, 1999; Biswas et al., 2005).

Rice-fish farming has been practiced in China and other Asian countries for many centuries to produce rice and fish together (Lu & Li, 2006). This combined farming ecosystem has the benefits of good crop production and helps promote biodiversity with low environmental risk (Xie et al., 2011). In rice-fish farming, the most commonly used fish are carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*) (Lu & Li, 2006). Loach, the other one of freshwater fishes that lives in ditches and streams around paddy fields. Loach was once widespread in the East Asian paddy fields, but is now discernibly less abundant (Tanaka, 1999). Many reasons have been posited for the decline in loach. Use of synthetic agricultural chemicals is one of the most important causes of reduced loach populations in rice paddies (Kano et al., 2010).

In our previous paper entitled as combined use of *Azolla* and loach suppressed paddy weed *Monochoria vaginalis* and increased rice yield without agrochemicals, we reported the first year results from an in situ container experiment in 2012 rice growth season (Cheng et al., 2015). The first year results showed a partial suppression of *M. vaginalis* and improved rice yield with *Azolla* or loach alone in the rice paddies contained a high density of *M. vaginalis* seeds. The combined use of *Azolla* and loach had more positive effect on weed suppression and 131% increase in rice yield over the control treatment. The previous study indicated that combined use of *Azolla* and loach as a valuable approach in organic rice farming, especially in organically farmed rice paddies with high densities of *M. vaginalis* seeds. For proving the continuous effects of using both *Azolla* and loach on rice growth and yield under organic rice farming, we continuously carried out the container experiment in the second rice growth season in the year of 2013.

Organic farming also is relevant to climate change because organic agricultural systems have inherent potential to reduce greenhouse gas emissions, and to enhance carbon sequestration in the soil (Scialabba & Muller-Lindenlauf, 2010; Lopes et al., 2011;

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

Gattinger et al., 2012). Gattinger et al. (2012) collected the data from 74 published papers with 209 pair organic-to-conventional farming for studying the soil organic carbon (SOC) changes by meta-analysis, the analysis study showed that organic farming has the potential to accumulate soil carbon. However, there was no rice paddy data in the sources for meta-analysis (Gattinger et al., 2012).

The objective of this continuous organic rice farming experiment was to prove the continuous effects of using both *Azolla* and loach on rice growth and yield and to examine the changes in SOC and total nitrogen (TN) after two rice growth seasons in 2013.

MATERIALS AND METHODS

Experimental site and climate

This research was firstly conducted in rice growth season in the year of 2012 at the Experiment Field, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata Prefecture, located in northeastern Japan (38°44′N, 139°50′E, 16 m elevation). The climate of this experimental site is temperate with heavy snow in winter. The rice growth in the experimental site is one crop per year in summer season from end of May to end of September. The daily average air temperature, monthly rainfall and sunshine time for rice growth season from June to September in 2012 and 2013 were showed in Table 1. Compared with the average values of 1981–2010 (30-year avg.) for air temperature, the rice growth season from June to September, 2012 was much hotter than the 30-year average, the rice growth season in 2013 was hotter than the 30-year average, but cooler than 2012. Compared with the average values of 1981–2010 (30-year avg.) for rainfall, and 1986–2010 (25-year avg.) for monthly average sunshine time, the 2012 rice growth season was much sunnier than the 25-year average, but 2013 season was similar as the 25-year average.

Table 1
Daily average air temperature, monthly rainfall and sunshine time for rice growth season from June to September in 2012, 2013 and the average values of 1981–2010 (30-year avg.) for air temperature and rainfall, and 1986–2010 (25-year avg.) for monthly average sunshine time. Data from the Japan Meteorological Agency.

Month	Daily average air temperature (^{o}C)			Monthly rainfall (mm)			Monthly sunshine time (hour)		
	Av. 30 years (1981-2010)	2012	2013	Av. 30 years (1981-2010)	2012	2013	Av. 25 years (1986-2010)	2012	2013
June	19.7	19.8	21.6	120.6	57.5	30.5	177.1	227.0	248.0
July	23.3	24.5	24.1	201.5	112.5	747.0	163.0	178.6	87.3
August	25.1	27.0	26.0	173.5	133.5	229.5	201.2	263.8	174.2
September	20.8	24.3	21.7	184.2	111.0	145.5	140.6	208.3	189.1
Average for 4 months	22.2	23.9	23.4	170.0	103.6	288.1	170.5	219.4	174.7

Experimental design for *Azolla* and loach treatments

We designed an in situ container experiment with 4 treatments and 3 replications. The 4 treatments were: (1) control, without any *Azolla* or loach; (2) *Azolla*, using the floating aquatic fern *Azolla filiculoides* (hereinafter we use *Azolla* to refer to both the treatment and to *Azolla filiculoides*); (3) loach (*Misgurnus anguillicaudatus*); and (4) combined *Azolla* and loach (abbreviated as Az+Lo). We chose to use containers instead of performing a field experiment because the *Azolla* we used is an introduced species (IRRI code FI 1001),

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

originally from the International Rice Research Institute in the Philippines. Without permission from relational governmental office, the introduced species could be not used in the field (Watanabe, 2006). The loach used in this study is *Misgurnus anguillicaudatus*, a native species bought from an aquaculture fish supplier (LP Farm, Inc., Akita, Japan). Loach size is about 6-8 cm long (Cheng et al., 2015).

Soil and container preparation for first rice season in 2012

The soil used in this experiment was a kind of alluvial collected from the plough layer (the top 15 cm of soil) of an organically managed rice field at the University Farm, located about 5 km from the experimental site on May, 2012. It contained 19.0 g kg^{-1} organic C and 1.63 g kg^{-1} total N, and the average pH was 5.3. The soil contained a high density of *M. vaginalis* seeds. The soil was air dried and sieved (5-mm mesh size) before use. One day before rice transplanting, 20.0 kg (15.0 kg dry soil equivalent) of soil mixed with 25 g of soybean oil cake (Olive Corp., Niigata, Japan) was placed into each rigid plastic container (53 cm long x 39 cm wide x 32 cm high) at 15 cm depth. The oil cake is an organic fertilizer made from the oily dregs of soybeans and it contained 382.4 g kg^{-1} organic C and 53.9 g kg^{-1} total N. The other management of rice cultivated can be referred Cheng et al. (2015).

Experimental management in second rice season in 2013

After harvested rice in first year on 24 September 2012 at 111 day after transplanting (DAT), the containers were left at in situ from September to May 2013. The *Azolla* and loach in the containers did not live through the winter. On 22 May 2013, the soils in all containers were mixed well with *Azolla* and loach residues left from last season. The dry weight of *Azolla* residues were about 30 g per containers in *Azolla* and Az+Lo treatments. The dry weight of died loaches were about 8 g per containers in loach and Az+Lo treatments (0.8 g dry weight per loach). Four rice plants (3 seedlings for 1 hill) were transplanted into each container on 28 May 2013. The rice cultivar was ‘Haenuki’ as same as last season in 2012. Two grams fresh weight of *Azolla* was inoculated into the water of each of the *Azolla* and Az+Lo containers; ten loaches were placed into each of the loach and Az+Lo containers after rice transplanting on the same day.

Measurements

Rice height, tiller number and top rice leaf greenness of each rice plant were measured once a week beginning 14 DAT. Rice was harvested on 15 September 2013 (110 DAT). Firstly, the fresh *Azolla* biomass in the *Azolla* and Az+Lo treatments and fresh paddy weed *M. vaginalis* aboveground biomass in all the treatments was taken out and washed by tap water, then fresh biomass were oven dried at 70 °C for calculating dry weight. Only the aboveground parts of rice plants were harvested. Four hills from each container were mixed together. Harvested rice ears and straw were separated from each other and air-dried in a glasshouse. After harvested over *M. vaginalis* and aboveground parts of rice plants, the other paddy weed *Eleocharis acicularis* in control containers was sampled and washed by tap water, then oven-dried at 80 °C for 3 days for calculating dry weight. *E. acicularis* was not emerged in the containers of *Azolla*, loach and Az+Lo treatments. Parts of oven-dried plant samples of rice, *Azolla* and weeds were ground for measuring C and N contents by CN-900 Analyzer (Sumika Chemical Analysis Service).

To measure grain yield, we air-dried the ears for 1 month, counted the number of panicles, and carefully threshed the grain. Grain was soaked in 1.06 g mL^{-1} saline ($(NH_4)_2SO_4$) solution and the numbers of sunken and floating grains were counted to determine the grain filling rate. The dry weight of sunken grain was determined after drying at 80 °C in a forced-air oven for 1 week. Grain yield was defined as the dry

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

weight of filled grain. Harvest index (HI) was calculated from the grain yield and the plant total aboveground dry weight.

Soil sampling and analysis

After plant sampling, the soil in all containers was divided to layers for analyzing SOC and TN contents. The top 0-2cm layer was termed as surface layers and from 2cm to bottom was termed as lower layer. The soils were air-dried and ground to 2mm for measuring SOC and TN contents by CN-900 Analyzer (Sumika Chemical Analysis Service).

Statistical analysis

Analysis of variance (ANOVA) was used to determine the statistical significance of measurement parameters among the 4 treatments. Least significant difference (LSD) values were calculated for each parameter for which significant differences were found ($P < 0.05$). The analysis was done with the SPSS 19 (SPSS Inc., Chicago, IL, USA) statistical package.

RESULTS

Rice plant height, tiller number, and SPAD value

Rice plant height increased steadily until the grain-filling stage (~77 DAT) in all 4 treatments. There was significant difference in rice plant height among the 4 treatments. At harvest, rice shoots of *Azolla* treatment was similar to the control, but was 6% higher in loach and 3% higher in the Az+Lo to the control treatment (Fig. 1a).

The tiller number reached a maximum (12.7 per plant) at 35 DAT in control treatment, at 42 DAT in *Azolla* (13.1), loach (22.4) and Az+Lo (22.7) treatments (Fig. 1b). Compared to control, maximum tiller numbers increased 77% in the loach containers and 79% in the Az+Lo containers (Fig. 1). At harvest (105 DAT), compared to control, the productive tiller numbers had increased by 19% in the *Azolla* treatment, 99% in the loach treatment and 103% in the Az+Lo treatment (Fig. 1b). Top rice leaf greenness, expressed as SPAD value, increased until 42 DAT as same as the tiller number changes in the loach and Az+Lo treatments (Fig. 1b & c). Until 63 DAT, the SPAD values were obviously larger in loach and Az+Lo treatments than those in control and *Azolla* treatments. After 63 DAT, SPAD values of control treatment were lower than those of the others 3 treatments (Fig. 1c).

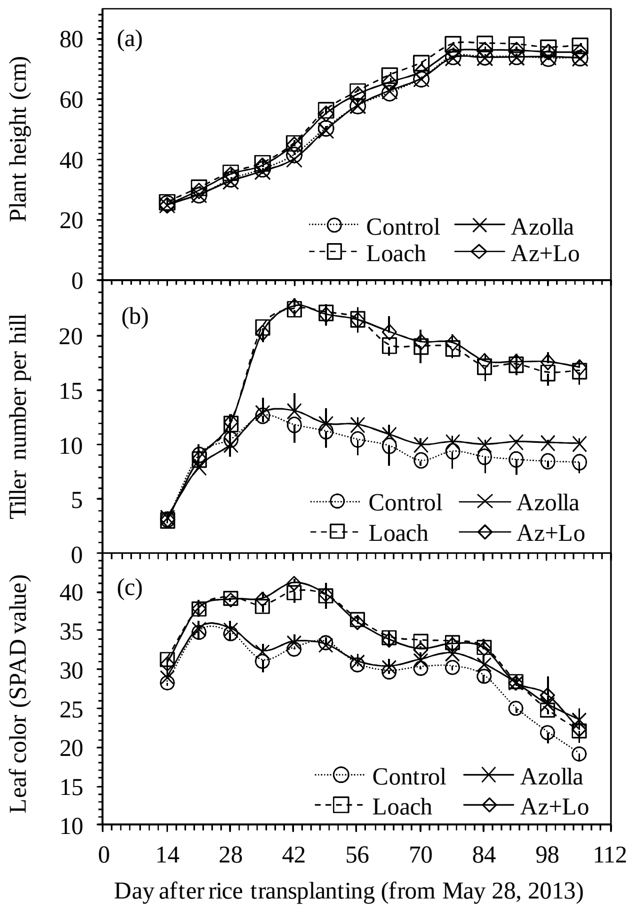

Fig. 1. Changes in plant heights (a), tiller numbers of rice plants (b) and leaf color in SPAD values (c) throughout the experiment among 4 treatments in 2013. Bars indicate standard deviation (n = 3).

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

Growth of rice, *Azolla* and weeds, and those biomass of at the harvest stage

Differed to last rice growth season in 2012, the *Azolla* grew slowly in both of *Azolla* and Az+Lo containers in the second season in 2013. Nine weeks later (63 DAT), both of *Azolla* and Az+Lo containers were covered by *Azolla*, it was 5 weeks later compared with last season in 2012. At harvest, the biomass of *Azolla* was 24.7 and 22.8 g per container for *Azolla* and Az+Lo treatments (Table 2). *M. vaginalis* was the predominant paddy weed species in the organic farm soil we used in this study. During the second rice growth season in 2013, the densities of *M. vaginalis* were thin in all 4 treatments containers and the dry biomass was ranged 0.49~2.87 g per container at harvest. However, *E. acicularis* became main paddy weed in control treatment containers, and the dry biomass was 48.2 g per container in 2013 (Table 2). The other paddy weeds, such as *Echinochloa oryzoides* and *Rotala indica* var. *Uiginosa*, can be ignored in all containers.

Table 2
The dry weights of rice (ear and straw), *Azolla* and paddy weeds (*M. vaginalis* and *E. acicularis*) among 4 treatments at harvest (September 15, 2013).

Treatments	Ear of rice	Straw of rice	Ear/Straw ratio of rice	*A. filiculoides*	*M. vaginalis*	*E. acicularis*
	(g container^{-1})			(g container^{-1})		
Control	43.4 b*	44.8 c	0.97 ab	–**	1.39 ab	48.2
Azolla	50.8 b	56.8 b	0.89 b	24.7 a	2.87 a	–
Loach	97.2 a	95.0 a	1.02 a	–	0.49 b	–
Az+Lo	97.4 a	91.5 a	1.07 a	22.8 a	0.56 b	–

*Values within each column followed by the different letters indicated significantly different among the treatments (P <0.05). **A. filiculoides was not inoculated in control and loach treatments, *E. acicularis* was only emerged in Control treatment, the other weeds weight was ignored in all treatments.

Aboveground biomass of rice was separated into ear and straw at harvest. Dry ear biomass was 43.4 g per container for control treatment, 50.8 g for *Azolla* treatment, 97.2 g for loach treatment, and 97.4 g for Az+Lo treatment (Table 2). Compared with control, the dry ear biomass was 17% higher in *Azolla* containers, 124% higher in loach containers, and 125% higher in Az+Lo containers. Compared with control, the dry straw biomass was 27% higher in *Azolla*, 112% higher in loach, and 104% higher in Az+Lo treatment. ANOVA and LSD statistical analysis showed there were significant differences between with and without loach used treatments (P < 0.05) Also there was significant difference in ear/straw ratio among all treatments. Compared with control, the ear/straw ratio decreased by 7% for *Azolla*, and increased 5% for loach, and 10% for Az+Lo treatments.

Grain yield and yield components

The average number of panicles per container was 32.3 for control treatment, 41.7 for *Azolla* treatment, 61.7 for loach treatment, and 66.0 for Az+Lo treatment (Table 3). Compared with control, the panicle number was 29% greater for *Azolla*, 91% greater for loach, and 104% greater for Az+Lo. The average number of spikelets per panicle was 52.7 for control, 48.0 for *Azolla*, 64.1 for loach, and 60.0 for Az+Lo (Table 3).

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

Compared with control, the number of spikelets per panicle was 9% less for *Azolla*, 22% greater for loach, and 14% greater for Az+Lo. The percentages of filled spikelets were between 93.6% and 95.0% and individual grain weights were between 24.4 and 25.0 mg among all treatments, without significant difference (Table 3). Overall, average grain yield per container was 39.7 g for control, 46.6 g for *Azolla*, 90.4 g for loach, and 91.6 g for Az+Lo. Compared with control, the grain yield was significantly higher in all treatments, by 17% for *Azolla*, 128% for loach, and 131% for Az+Lo (Table 3). Compared with 2012 season, the grain yields decreased in 2013 season in all treatments (Cheng et al., 2015). The harvest index was 45.0% for control, 43.3% for *Azolla*, 47.0% for loach, and 48.5% for Az+Lo in 2013 season (Table 3).

Table 3
Effects of *Azolla* and loach application on yield and its components of Haenuki rice crop, and harvest index among 4 treatments in 2013 year.

Treatments	Panicle No. per container	Spikelet No. per panicle	Filled Spikelet (%)	Individual Grain weight (mg)	Grain yield (g per container)	Harvest index (%)
Control	32.3 c*	52.7 b	94.0 a	24.8 a	39.7 b	45.0 b
Azolla	41.7 b	48.0 b	93.6 a	25.0 a	46.6 b	43.3 b
Loach	61.7 a	64.1 a	93.6 a	24.4 a	90.4 a	47.0 a
Az+Lo	66.0 a	60.0 a	95.0 a	24.4 a	91.6 a	48.5 a

*Values within each column followed by the different letters indicated significantly different among the treatments (P <0.05)

Table 4
The N contents of rice (ear and straw), *Azolla* and paddy weeds (*M. vaginalis* and *E. acicularis*) among 4 treatments at harvest (September 15, 2013).

Treatments	Ear of rice	Straw of rice	*A. filiculoides*	*M. vaginalis*	*E. acicularis*
	(%)				
Control	0.92 b*	0.45 b	–**	1.49 a	1.22
Azolla	0.99 a	0.61 a	3.44 a	1.93 a	–
Loach	0.98 a	0.53 ab	–	1.59 a	–
Az+Lo	0.98 a	0.65 a	3.00 b	1.91 a	–

*Values within each column followed by the different letters indicated significantly different among the treatments (P <0.05). **A. filiculoides was not inoculated in control and loach treatments, *E. acicularis* was only emerged in Control treatment, the other weeds weight was ignored in all treatments.

The N contents and N absorptions of rice, *Azolla* and weeds at the harvest

The N content of rice ears at harvest was 0.92% for control, significantly lower than 0.98~0.99% for *Azolla*, loach, and Az+Lo treatments (Table 4). The N content of rice

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

straws was 0.46% for control, 0.61% for *Azolla*, 0.53% for loach, and 0.65 for Az+Lo. Compared with control, the N content of rice straws was significantly higher in *Azolla* and Az+Lo treatments, was not significantly higher in loach treatment (Table 4). The N content of *Azolla* was 3.44 and 3.00% for *Azolla* and Az+Lo treatments with significant difference (Table 4). The N content of *M. vaginalis* was 1.49~1.93%, no significant difference among 4 treatments. The N content of *E. acicularis* was 1.22%, which was emerged in control treatment only (Table 4).

The N absorption of aboveground of rice was 0.604 g per container for control, 0.841 g for *Azolla*, 1.456 g for loach, and 1.555 g for Az+Lo. Compared with control, the N absorption by rice plant in aboveground biomass was significantly higher in *Azolla* or/and loach treatments, by 39% for *Azolla*, 141% for loach, and 157% for Az+Lo (Table 5). The N absorption of *Azolla* (including part N fixation by *Azolla* itself) was 0.848 and 0.681 g per container for *Azolla* and Az+Lo treatments with significant difference (Table 5). The N amount of *M. vaginalis* was around 0.008~0.053 g per container, only *Azolla* treatment was significantly higher than others. The N amount of *E. acicularis* was 0.587 g per container, which was closed to the N absorption by aboveground biomass of rice in control treatment (Table 5).

Table 5
The N absorption of rice, *Azolla* and paddy weeds (*M. vaginalis* and *E. acicularis*) among 4 treatments at harvest, the roots were excepted (September 15, 2013).

Treatments	Ear of rice	Straw of rice	Aboveground of rice	*A. filiculoides*	*M. vaginalis*	*E. acicularis*	Total of all vegetations
	($g\ N\ container^{-1}$)						
Control	0.401 b*	0.203 c	0.604 c	– **	0.021 b	0.587	1.212 c
Azolla	0.499 b	0.342 b	0.841 b	0.848 a	0.053 a	–	1.743 b
Loach	0.950 a	0.506 a	1.456 a	–	0.008 b	–	1.464 c
Az+Lo	0.959 a	0.597 a	1.555 a	0.681 b	0.011 b	–	2.248 a

*Values within each column followed by the different letters indicated significantly different among the treatments (P <0.05). ***A. filiculoides* was not inoculated in control and loach treatments, *E. acicularis* was only emerged in Control treatment, the other weeds weight was ignored in all treatments.

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

Table 6
The changes in soil organic C (SOC), total N (TN) contents and C/N ratio in the surface (0-2cm) and lower layers (2cm to bottom) of the soils among 4 treatments containers at harvest (September 15, 2013)*.

Treatments	SOC (g kg^{-1})		Ratio of surface to lower	TN (g kg^{-1})		Ratio of surface to lower	C/N		Ratio of surface to lower
	Surface	Lower		Surface	Lower		Surface	Lower	
Control	22.9 ^{b**}	18.8 ^{b}	1.22	2.04 ^{b}	1.58 ^{a}	1.29	11.2 ^{b}	11.9 ^{a}	0.94
Azolla	25.6 ^{ab}	20.5 ^{a}	1.25	2.16 ^{ab}	1.69 ^{a}	1.28	11.9 ^{a}	12.1 ^{a}	0.98
Loach	23.7 ^{b}	19.0 ^{ab}	1.24	2.11 ^{ab}	1.59 ^{a}	1.33	11.2 ^{b}	11.9 ^{a}	0.94
Az+Lo	26.9 ^{a}	19.8 ^{a}	1.36	2.32 ^{a}	1.63 ^{a}	1.42	11.6 ^{a}	12.1 ^{a}	0.96

*The original soil used for this experiment had SOC at 19.0 g kg^{-1} and TN at 1.63 g kg^{-1}. **Values within each column followed by the different letters indicated significantly different among the treatments (P <0.05).

The changes in soil SOC and TN contents after 2 rice seasons

There were large differences of SOC and TN between surface layer (0-2cm) and lower layer (2cm to bottom) soils in the containers among 4 treatments at harvest in 2013 after 2 rice growth seasons. SOC of surface layer soil was 22% greater than that of lower layer for control, 25% greater for *Azolla*, 24% greater for loach, and 36% greater for Az+Lo. Similarly, TN of surface layer soil was 29% greater than that of lower layer for control, 28% greater for *Azolla*, 33% greater for loach, and 42% greater for Az+Lo (Table 6). Compared with the original soil used for this experiment in 2012 (19.0 g kg^{-1} SOC and 1.63 g kg^{-1} TN), both SOC and TN of lower layer soils were increased in *Azolla* treatment. The ratio of C and N (C/N) was around 11~12 for both surface and lower layer soils among 4 treatments, while the C/N of surface layer soils were obvious smaller than that of lower layer (Table 6).

DISCUSSION

Use of *Azolla* and loach suppressed paddy weeds under organic farming practice in the second rice season in 2013

In our previous paper, we reported that combined use of *Azolla* and loach totally suppressed *M. vaginalis* emergence in the first rice growth season in 2012 (Cheng et al., 2015). In the first season, many *M. vaginalis* seedlings emerged from the underwater soil surface during the first week after rice transplanting at control containers due to the original soil contained a high density of *M. vaginalis* seeds. However, the main paddy weed was *E. acicularis* instead of *M. vaginalis* in the same control containers in the second rice season in 2013. The weight of *E. acicularis* was 48.2 g per container, and was equal to 55% of rice aboveground biomass in the containers (Table 2). It indicated that the main paddy weeds can be changed in different rice growth season.

As same as the first rice season in 2012, the emergence of paddy weed *M. vaginalis* was partially suppressed by each *Azolla* or loach treatments in the same containers during the second rice growth season in 2013, however, the dry biomass of *M. vaginalis* was quite small (0.49~2.87 g per container) (Table 2), if it was compared with the dry biomassin the first season in 2012 (18.2~26.3 g per container) (Cheng et al., 2015). It could be considered that the seeds of *M. vaginalis* were decreased in the second rice growth season in 2013 due to most of seeds germinated in the

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

first season in 2012 and no new *M. vaginalis* seeds can be produced in 2012 since the *M. vaginalis* plants were incorporated in the surface soil early. It indicated that the paddy weeds emergences can be decreased by continuous organic farming practice if the seeds bank of paddy weeds were controlled by previous rice growth season.

It was unlike the first rice growth season 2012, combined use of *Azolla* and loach did not totally suppress emergences of *M. vaginalis* in Az+Lo containers in the second rice season in 2013. The reason was that *Azolla* growth in 2013 season was slower than that in 2012 due to lower temperature, lower sunshine time and higher rainfall in second rice growth in 2013 compared with those in 2012 (Table 1).

Effect of loach was larger than *Azolla* on rice yield in the second rice season in 2013

In our previous paper, we reported that rice yield was improved by single *Azolla* or loach used, while combined use of *Azolla* and loach had more positive effect on rice yield in the first rice growth season in 2012 (Cheng et al., 2015). However, the effect of loach used was larger than *Azolla* used on rice yield in the second rice growth season in 2013 (Table 3). Compared with the first season in 2012, the rice yields were decreased at 49% for control, at 56% for *Azolla*, 35% for loach, and 50% for Az+Lo in the second season in 2013 (Table 3 and Cheng et al., 2015). Both reasons could be thought for the rice yield decreases. The first reason was that we did not apply the soybean oil cake as organic manure for the second rice growth season in 2013. So the soil fertilities were lower the first season in 2012. The soybean oil cake was applied at 25 g per container (equal to 1.35 g N per container or 6.52 g N/m^2) in first season in 2012, which provided rice nutrients during early rice growth stage for improving both panicle number per plant and spikelet number per panicle (Matsushima & Tanaka, 1960; Cheng et al., 2009). In the second season in 2013, we expected that the soil fertilities would be improved by plowing the *Azolla* biomass and loach debris from the last season, but the second season results indicated that *Azolla* biomass (about 1g N per container, calculated by the biomass in the first season 2012 and the N content in the second season 2013) could be not as quick-acting fertilizer for rice growth. The second reason for lower rice yield in 2013 than that in 2012 was due to lower temperature and sunshine time in 2013 compared with those in 2012.

If we compared the rice yield among 4 treatments in 2013, the grain yield was increased at 128 % and 131% at loach and Az+Lo treatments to the control (Table 3). The effect of loach used was significantly larger than *Azolla* on rice yield in the second rice season in 2013. Three reasons could be considered for the rice yield increase by loaches used. The first reason was that the died loaches biomass from 2012 season become a quick-acting fertilizer for rice growth for 2013 season, because 10 died loaches had about 8 g dry biomass with high N content. The N content of loaches was 11.9% and C/N was 3.58. The died loaches left in the containers for both loach and Az+Lo treatments equal to 0.95 g N per container or 4.61 g N/m^2. The quick-acting N fertilizer from died loaches biomass significantly increased panicle number per plant and spikelet number per panicle in the early rice growth stage in 2013 (Fig. 1b; Table 3). The second reason was that the living loaches applying in 2013 season again help to loosen the surface soil and their movement increases the oxygen content of soil, as well as enhancing microbial activity and stimulating soil and additional organic matter (died loaches, rice stubbles and *Azolla* in Az+Lo treatment only) decomposition and mineralization. The third reason was that loach excreta is a fertilizer that is rapidly available for absorption by rice through mechanisms similar to those found in the carp and tilapia rice–fish culture systems still used in China and other Asian countries (Lu & Li, 2006, Xie et al., 2011).

Rice yield is determined by panicle number per land area, spikelet number per panicle, filled spikelet percentage, and

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

individual grain weight. Among the 4 components of rice yield, filled spikelet percentage and individual grain weight were not affected by single or combined use of *Azolla* and loach in both 2012 and 2013 seasons (Table 3; Cheng et al., 2015). It indicated that suppressing paddy weeds emergences and improving rice nutrients during the early rice growth stage was very important for organic rice farming.

Rice growth and grain yield depend on plant N accumulation, and rice photosynthesis is sensitive to leaf N concentration (Kobayashi et al., 2006). *Azolla* has symbiotic N-fixing cyanobacteria (*Anabaena azollae*) within its leaves and has been cultivated for many centuries in traditional rice paddies in southern China and northern Vietnam as a "green manure" to improve N availability to rice (Watanabe & Liu, 1992; Wagner, 1997). As with *Azolla* alone in second rice season 2013, the rice yield was light increased compared with control, but it was not significant (Table 3). Though *Azolla* contained high N content (>3%, Table 4), the decomposition of *Azolla* biomass and its N mineralization were not so quickly as our expected. It looked like that the *Azolla* biomass plowed to soil from first season in 2012 was left in the soil until harvest stage in second rice growth season in 2013 (Table 6, see detail below).

Effects of *Azolla* and loach used on the changes in soil SOC and TN contents

Our results showed that there were large differences of SOC and TN between surface layers (0-2cm) and lower layers (2cm to bottom) in the containers among 4 treatments at harvest in 2013. It indicated that organic matter was accumulated in the surface layer during one rice growth season since the surface and lower layers were mixed together before rice transplanting in both rice growth seasons. The accumulation of organic matter was from many processes, such as rice root secretion, inputs of paddy weeds and *Azolla* residues, and loaches excretions. The amounts of SOC and TN in surface layer soils of 4 treatments were larger than those of the original soil used for this experiment in 2012 (19.0 g kg^{-1} SOC and 1.63 g kg^{-1} TN) also indicated the surface layer of submerged rice paddy has high net primary productivity and the rate of organic matter destruction is slower than its accumulation (Sahrawat, 2003).

We have discussed in previous section of this paper that rice growth and yield were not affected by the plowing *Azolla* to soil in second rice growth season in 2013, though the N amount was about 1g N per container (equal to 5g N/m^{-2}) from plowed *Azolla*. Here, the soil analysis data showed that both SOC and TN contents of lower layer soils in *Azolla* containers were highest among 4 treatments and the values were larger than those of original soil. This fact also indicated that the decomposition of *Azolla* biomass and its N mineralization were slowly during the second rice growth season in 2013.

CONCLUSIONS

Compared with the first rice growth season in 2012, the second season in 2013 showed that paddy weed of *M. vaginalis* emergence was decreased in control, *Azolla* and loach treatments. *E. acicularis* became main paddy weed in control containers in the second rice growth season in 2013 from *M. vaginalis* in 2012. Rice yields were decreased in the second rice growth season in 2013 due to cooler weather and no organic manure application compared with the first season in 2012. The decomposition of plowed *Azolla* biomass and its N mineralization were not so quickly to improve rice nutrients, but it increased the soil organic matter accumulation with high SOC and TN contents in both surface and lower layers of rice paddy. Both use of *Azolla* and loach are effective for organic rice farming, but loach used was more effective than *Azolla* used in the second rice growth season in 2013. The two years study

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

indicated that combined use of *Azolla* and loach can meet two of the greatest challenges in organic rice production: providing effective weed control and improving rice nutrition without agrochemicals.

ACKNOWLEDGEMENTS

This research was partly funded by the Center of Excellence Program grant from Yamagata University (YU-COE, No. E23-6) and Grants-in-Aid for Scientific Research (No. 23405045) from the Japan Society for the Promotion of Science.

REFERENCES

Biswas, M., Sultana, S., Shimozawa, H., Nakagoshi, N., 2005. Effect of *Azolla* species on weed emergence in a rice paddy ecosystem. Weed Biol Manag. 5, 176–183.

Brown, M.E., Funk, C.C., 2008. Food security under climate change. Science 319, 580–581.

Cheng, W., Sakai, H., Yagi, K., Hasegawa, T. 2009. Interactions of elevated [CO_2] and night temperature on rice growth and yield. Agr Forest Meteorol. 149, 51–58.

Cheng, W., Sakai, H., Matsushima, M., Yagi, K., Hasegawa, T., 2010. Response of the floating aquatic fern *Azolla filiculoides* to elevated CO_2, temperature, and phosphorus levels. Hydrobiologia 656, 5–14.

Cheng, W., Okamoto, Y., Takei, M., Tawaraya, K., Yasuda, H., 2015. Combined use of *Azolla* and loach suppressed weed *Monochoria vaginalis* and increased rice yield without agrochemicals. Org Agr. 5, 1–10.

Datta, B.K., Nayak, D.R., Sinhababu, D.P., Adhya, T.K., 2009. Methane and nitrous oxide emissions from an integrated rainfed rice–fish farming system of Eastern India. Agr Ecosyst Environ. 129, 228–237.

De Macale, M.A.R., Vlek, P.L.G., 2004. The role of *Azolla* cover in improving the nitrogen use efficiency of lowland rice. Plant Soil 263, 311–321.

FAO, 2015. FAOSTAT database. http://faostat.fao.org/. (accessed February, 2015)

Gattinger, A., Muller, A., Haeni, M., Skinner, C., Fliessbach, A., Buchmann, N., Mäder, P., Stolze, M., Smith, P., Scialabba N.E., Niggli, U., 2012. Enhanced top soil carbon stocks under organic farming. Proc Natl Acad Sci USA 109, 18226–18231

Janiya, J.D., Moody, K., 1984. Use of *Azolla* to suppress weds in transplanted rice. Trop Pest Manag. 30, 1–6.

Kano, Y., Kawaguchi, Y., Yamashita, T., Shimatani, Y., 2010. Distribution of the oriental weather loach, *Misgurnus anguillicaudatus*, in paddy fields and its implications for conservation in Sado Island, Japan. Ichthyol Res. 57, 180–188.

Kobayashi, K., Okada, M., Kim, H.Y., Lieffering, M., Miura, S., Hasegawa, T., 2006. Paddy Rice Responses to Free-Air [CO_2] Enrichment. In: Nösberger, J., S.P. Long, R.J. Norby, M. Stitt, G.R. Hendrey & H. Blum (eds), Managed ecosystems and CO_2: case studies, processes, and perspectives. Springer, Berlin Heidelberg New York, (Ecological studies, vol. 187), pp. 87–104.

Lopes, A.R., Faria, C., Prieto-Fernández, A., Trasar-Cepeda, C., Manaia, C.M., Nunes, O.C., 2011. Comparative study of the microbial diversity of bulk paddy soil of 2 rice fields subjected to organic and conventional farming. Soil Biol Biochem. 43, 115–125.

Lu, J., Li, X., 2006. Review of rice–fish-farming systems in China – One of the Globally Important Ingenious Agricultural Heritage Systems (GIAHS). Aquaculture 260, 106–113.

Mandal, B., Vlek, P.L.G., Mandal, L.N., 1999. Beneficial effects of blue-green algae and *Azolla*, excluding supplying nitrogen, on wetland rice fields: a review. Biol Fert Soils 28, 329–342.

Matsushima, S., Tanaka, T., 1960. Analysis of developmental factors determining yields and its application to yield prediction and culture improvement of a lowland rice. IV. Early discrimination of non-fertilized rice-

Available on line at:
http://ijwem.unlam.ac.id/index.php/ijwem
ISSN: 2354-5844

grains. Proc Jpn Crop Sci Soc. 28, 365–366 (in Japanese).

Sahoo, S.K., Datta, B.K., 1999. Weed control by *Azolla* in rice (*Oryza sativa*) fields of South 24-Parganas, West Bengal. Indian J Agr Sci. 69, 283–284.

Sahrawat,K.L., 2003. Organic matter accumulation in submerged soils. Adv Agr. 81, 169–201

Scialabba, N.E.H., Muller-Lindenlauf, M., 2010. Organic agriculture and climate change. Renew Agr Food Syst. 25, 158–169.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., Polasky, S., 2002. Agricultural sustainability and intensive production practices. Nature 418, 671–677.

Xie, J., Hu, L., Tang, J., Wu, X., Li, N., Yuan, Y., Yang, H., Zhang, J., Luo, S., Chen, X., 2011. Ecological mechanisms underlying the sustainability of the agricultural heritage rice–fish coculture system. Proc Natl Acad Sci USA 108, E1381–E1387.

Seufert, V., Ramankutty, N., Foley, J., 2012. Comparing the yields of organic and conventional agriculture. Nature 485, 229–232.

Smil, V., Kobayashi, K., 2012. Japan’s dietary transition and its impacts. The MIT Press, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142 USA.

Tanaka, M., 1999. Influence of different aquatic habitats on distribution and population density of *Misgurnus anguillicaudatus* in paddy fields. Jpn J Ichthyol 46, 75–81 (In Japanese).

Wagner, G.M., 1997. *Azolla*: A review of its biology and utilization. Bot Rev. 63, 1–26.

Watanabe, I., Liu, C.C., 1992. Improving nitrogen-fixing systems and integrating them into sustainable rice farming. Plant Soil 141, 57–67.

Watanabe, I., 2006. Whither *Azolla* use goes? Regulation by invasive alien species act. J Weed Sci Technol. 51, 178–184 (in Japanese).