

## Allelopathic effects of *Fimbristylis miliacea* on the physiological activities of five Malaysian rice varieties

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### Abstract

*Fimbristylis miliacea*, a sedge having allelopathic potential is a dominant weed in the rice fields. Experiments were carried out in the laboratory and greenhouse to evaluate the allelopathic effects of *F. miliacea* on the physiological activities of five Malaysian rice varieties namely MR211, MRQ74, MR220, MR84 and MR232. The allelopathic effects of *F. miliacea* were assessed based on the chlorophyll and melondialdehyde (MDA) content, the activity of antioxidant enzymes namely catalase (CAT) and peroxidase (POD) of the rice seedlings, because physiological activities are hindered by allelopathy. Three concentrations of the aqueous extract of the weed (12.5 gl<sup>-1</sup>, 25 gl<sup>-1</sup> and 50 gl<sup>-1</sup>) and weed debris (5, 10 and 20 g dry debris / 1000 g soil) were used in the experiments carried out in the laboratory and green house, respectively. Among the rice varieties tested, it was observed that the MDA content of MRQ74 increased with increasing concentrations of the aqueous extract. The chlorophyll content of MRQ74 showed the highest reduction (64.5% of the control) in the presence of *F. miliacea* debris compared to that of the other four varieties, indicating the susceptibility of the rice variety to weed allelopathy. The results showed that there was an imbalance in the activity of catalase (CAT) and peroxidase (POD) in the rice seedlings due to the allelopathic effect of the weed species. The mitotic index of onion root treated with *F. miliacea* aqueous extract decreased compared to that of the control. The results clearly showed that *F. miliacea* has definite allelopathic effects on the rice variety, MRQ74 (compared to the other varieties tested).

**Keywords:** Phytotoxic, aqueous extract, *Fimbristylis miliacea*, Chlorophyll, Mitotic index.

**Abbreviations:** MDA\_Melondialdehyde; CAT\_Catalase; POD\_Peroxidase; ROS\_Reactive oxygen species; TBA\_Thiobarbituric acid; TCA\_Trichloro acetic acid; MARDI\_Malaysian Agricultural Research and Development Institute.

### Introduction

*F. miliacea*, a sedge is a dominant weed in rice fields, especially in South-East Asia (Moody, 1989). In Malaysia, it is the fifth and third most serious weed in the rice growing areas of Muda and Besut respectively (Karim et al., 2004). *F. miliacea* is also reported as a dominant weed species in rice fields (Azmi and Mashhor, 1995; Begum et al., 2005) with emergence density of 54 - 3074 plants m<sup>-2</sup> (Watanabe et al., 1997). Ismail and Siddique (2012b) reported that, *F. miliacea* showed phytotoxic inhibition on the growth parameters of the rice plants. Two allelochemicals that have been identified and can be used as biopesticides are *hexanedioic acid dioctyl ester and di-n-octyl phthalate* (Ismail and Siddique, 2012a). Although allelopathy has ecological and agronomic importance, relatively little is known concerning the mechanism or the adaptive strategies of plants in their defense against allelochemicals (Bais et al., 2006). It has recently been suggested that allelochemicals may influence the growth of neighbouring plants by the initiation of oxidative stress. Allelochemical stress can cause oxidative damage, as evidenced by enhanced activity of ROS (Reactive oxygen species)-scavenging enzymes and causing an increased degree of membrane lipid peroxidation (Lara-Nunez et al., 2006; Ye et al., 2006). It has also been postulated that the allelopathic effect might lead to an imbalance between antioxidant defenses and the amount of reactive oxygen species (ROS), resulting in oxidative stress (Romero-Romero et al., 2005). The intracellular level of

hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is regulated by a wide range of enzyme, the most important of which is catalase (CAT) (Blokchina et al., 2003). Peroxidase (POD) activities in cucumber root were found to increase significantly after exposure to phytotoxic agents (Yu et al., 2003). The decrease in chlorophyll synthesis is a common response of plants to allelochemicals, and this might be a subsequent response of the plant to these chemicals besides cellular damage. Allelochemicals adversely affect chlorophyll accumulation by interfering in chlorophyll biosynthesis and/or destruction, consequently leading to poor plant growth (Tanveer et al., 2008). A cytogenetic bioassay in plants can be used for the analysis of chromosomal damage or disturbances in the cell cycle, and this method has the benefit of allowing for the determination of a detailed action mechanism on genetic material by a chemical compound that penetrates the cells during several subsequent cellular cycles. Due to the suitable chromosomal features, onion is considered one of the best biological models for studying chromosomal damage or disturbances in the cell cycle (Charoenying et al., 2010). In previous experiments, *F. miliacea* showed inhibitory effects on rice seedling growth and allelochemicals are identified from this weed species. To date, the allelopathic effect of *F. miliacea* on the chlorophyll and melondialdehyde (MDA) content, the activity of antioxidant enzymes namely catalase (CAT) and peroxidase (POD) and the mitotic index of the bioassay species have not been reported. The current study

was conducted to determine the allelopathic effects of *F. miliacea* on the chlorophyll and melondialdehyde (MDA) content, the activity of antioxidant enzymes (catalase and peroxidase) and the mitotic index of bioassay species.

## Results and Discussion

### Effect on MDA content

Fig 1A. shows the phytotoxic effect of *F. miliacea* on the MDA content of rice plants. The MDA content of MRQ74 has been increased at all levels of concentration of the weed extract compared to the control. The content of MDA increased at half strength than at quarter strength of the *F. miliacea* extract and decreased at full strength but still higher than the control. All the other four rice varieties except MRQ74 treated with the water extract of *F. miliacea* did not show the increasing trend of MDA content but was rather decreasing. The formation of free radicals in the cells would result in damage to cell membranes due to lipid peroxidation. Thus, the level of MDA, produced during lipid peroxidation, is a good indicator of oxidative damage that could be occurring within the cells (Masia, 2003). In the present study, enhanced MDA content suggests that the aqueous extract of *F. miliacea* may contain substances which probably induce oxidative stress, and, as a result, disrupt cellular membrane structure, and cause loss of cellular integrity in the MRQ74 leaf. Lara-Nunez et al. (2006) reported that increased MDA content is associated with increased O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> production following biotic and abiotic stresses. Accumulation of H<sub>2</sub>O<sub>2</sub> in the MRQ74 leaves in response to *F. miliacea* treatment enhances lipid peroxidation and caused severe oxidative stress resulting in disruption of metabolic activities in the cell.

### Effect on enzyme activity

The POD activity in all the rice varieties treated with the aqueous extract of *F. miliacea* was higher than that of the control at all the concentrations tested with the exception of MR84 at quarter strength (Fig. 1B). The activity of POD in MR211 increased at quarter and half strength of the extract compared to the control. The POD activity in MR211 has been decreased at full strength of the aqueous extract of *F. miliacea* but was still higher than that of the control. The aqueous extract of *F. miliacea* increased the POD activity in MR220 and MR232 at quarter strength. The activity of POD in both the varieties decreased at half and full strength of the extract but still higher than that of the control except MR232 at full strength. The POD activity of MRQ74 increased at quarter strength then decreased slightly at half strength but increased at full strength of the *F. miliacea* extract. The CAT activity in the rice seedlings of MR211 treated with the aqueous extract of *F. miliacea* decreased at half and full strength of the concentration (Fig. 1C). The activity of CAT in MRQ74 increased with increasing concentration of the extract of *F. miliacea* at quarter and half strength (compared to the control) but decreased slightly at full strength. At quarter strength the CAT activity in MR220 was higher than that of the control but it was lower at the half and full strength. The CAT activity in MR84 was significantly lower at quarter strength but increased with increasing concentration of the *F. miliacea* aqueous extract. However the CAT activity increased at full strength and it was observed to be slightly higher than that of the control. The trend of the CAT activity of MR232 gradually increased with increasing concentration of the weed extract. The results

shown in Figure 1A and 1B are consistent with those of Cruz-Ortega et al. (2002), who reported that allelochemicals cause increase in the activity of antioxidant enzymes and suggest that increased induction of these enzymes is necessary to prevent lipid peroxidation (i.e., to counter the higher MDA and H<sub>2</sub>O<sub>2</sub> levels in leaves). The results for variety MRQ74 are consistent with those of Batish et al. (2006), who showed that the exposure of mung bean to 2-benzoxazolinone caused an increase in the activity of catalase (CAT). An increase of POD activity in response to allelochemicals has also been reported by Yu et al. (2003) for cucumber root. The POD activity of MR220 and MR232, CAT activity of MRQ74 and MR220 when treated with *F. miliacea* extract showed significant increase at lower concentrations and this finding is consistent to that observed for other allelochemicals as reported by Qian et al. (2009), except that the trend was decreasing activity with increasing concentration of the extract. It can be speculated that at high concentration, the allelochemical might directly inhibit oxidizing enzymes in some way, leaving the plant vulnerable to oxidative damage as reported by Qian et al. (2009).

### Effect on chlorophyll content

Fig 2 shows the phytotoxic effect of *F. miliacea* on the chlorophyll content of the 5 rice varieties (MR211, MRQ74, MR220, MR84 and MR232). The debris of *F. miliacea* reduced the chlorophyll content of all the rice varieties at all concentrations except for MR220 at quarter strength (5 g/ kg soil). It did not have any adverse effect on the chlorophyll content of MR220 at quarter strength but rather it produced a slight stimulation. The chlorophyll content of the other four rice varieties namely MR211, MRQ74, MR84 and MR232 was adversely affected by the debris of *F. miliacea* because significant chlorophyll content reduction started at quarter strength concentration (5 g/ kg soil) of the debris. The chlorophyll content of the rice seedlings decreased progressively when exposed to increasing concentrations of *F. miliacea* tissue. The chlorophyll content of MRQ74 was greatly reduced (64.5% compared to the control) by the debris of *F. miliacea*. Decrease in chlorophyll content in the presence of allelochemicals has been reported by Baziramakenga et al. (1994), Patterson (1981) and Zeng et al. (2001). Since chlorophyll content is closely related to plant dry matter production (Bottery and Bozzell, 1977), reduction in leaf chlorophyll content would cause decreased photosynthesis and hence total plant growth.

### Effect on the Mitotic index of onion root

Table 1 shows the mitotic index and mitotic phases of onion root-meristem cells exposed to different concentrations of *F. miliacea* aqueous extracts. After incubation for 24 h, the mitotic index of the onion root decreased when treated at half and full strength of the aqueous extract of *F. miliacea*. But at quarter strength (5 g/1000 ml) the mitotic index of the onion root increased (compared to the control). Many studies have shown that allelochemicals stimulate growth at lower concentrations and inhibit growth at higher concentrations (Mandal, 2001; Ismail and Chong, 2009). The results of this study indicated that the mode of action of the toxicity of the *F. miliacea* extract involved disturbance of the mitotic processes. A similar observation caused by *Jasminum officinale* L. f. var. *grandiflorum* (L.) Kob on bioassay plants was reported by Montinee et al. (2010). This reduction in mitotic index suggests that exposure to the *F. miliacea* extract leads to cell cycle disturbances causing a decrease in

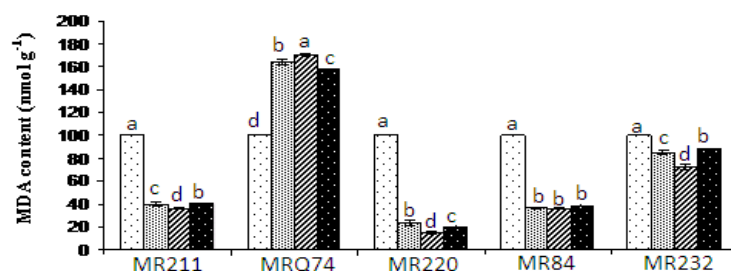
**Table 1.** Phytotoxic effects of the aqueous extract of *F. miliacea* on the mitotic index of onion (*A. cepa*) root.

Extract concentration	Examined total cells	Cells in mitosis	Mitotic index	Interphase	Mitotic phase (%)			
					Prophase	Metaphase	Anaphase	Telophase
Control (o)	4018	987	24.6b	3248	68.4b	22.0c	5.3c	4.4c
Quarter (12.5 g l <sup>-1</sup> )	3403	1022	30.0a	2676	77.8a	21.2c	5.1c	4.2c
Half (25.0 g l <sup>-1</sup> )	4503	772	17.1c	4088	61.5c	36.8a	8.8a	7.3a
Full (50.0 g l <sup>-1</sup> )	4642	589	12.7d	3793	32.1d	28.1b	6.7b	5.6b

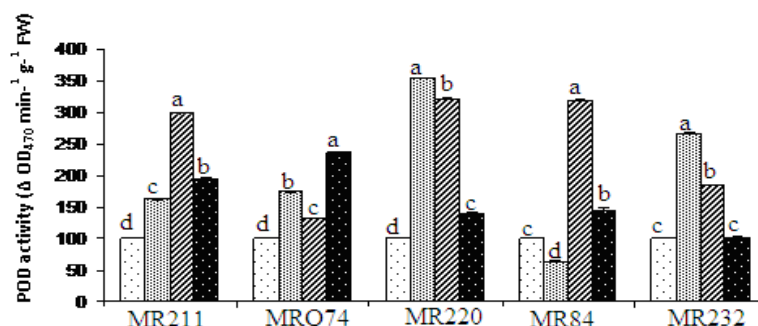
Means within columns followed by same alphabet are not significantly different ( $p \leq 0.05$ ).

□ Control (0)    ▨ Quarter (12.5 g l<sup>-1</sup>)    ▩ Half (25.0 g l<sup>-1</sup>)    ■ Full (50.0 g l<sup>-1</sup>)

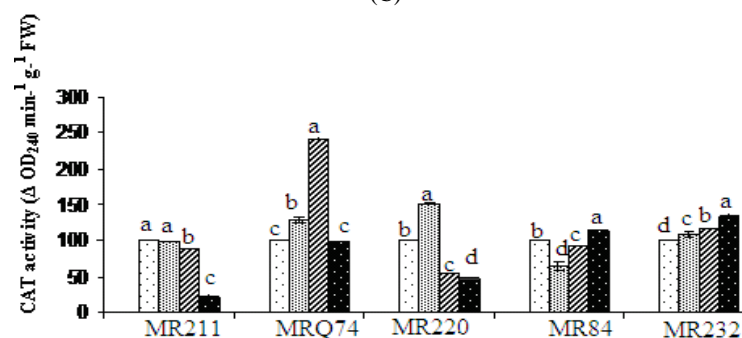
(A)



(B)



(C)



Rice varieties

Means within clusters followed by same alphabet are not significantly different ( $p \leq 0.05$ ).

**Fig 1.** Phytotoxic effects of the aqueous extract of *F. miliacea* on the (A) MDA content (B) POD activity and (C) CAT activity of 5 rice varieties.

cell numbers entering mitotic division. Due to the reduced number of dividing cells, it can be postulated that *F. miliacea* might have a negative effect on cell division (as seen in *Allium cepa* L. root) and possibly be involved in blocking the DNA or protein synthesis required for the normal cell division process (Schulze and Kirscher, 1986).

## Materials and methods

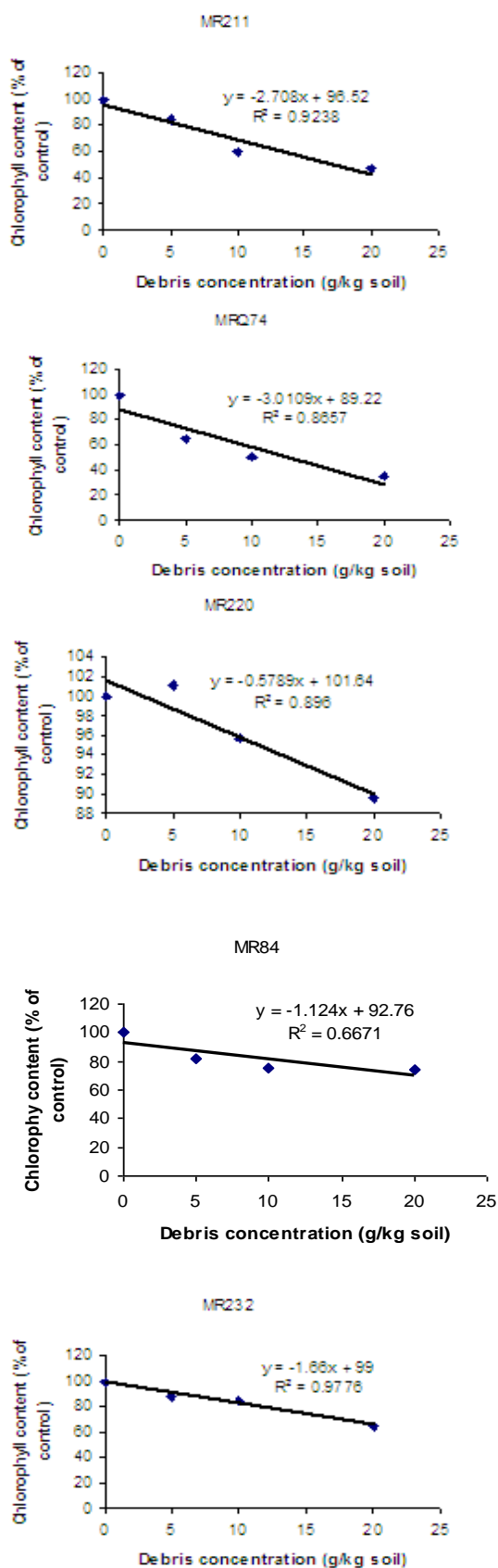
### Plant materials

*Fimbristylis miliacea* plants at vegetative growth stage were collected from the Tanjung Karang rice growing area in

Selangor, Malaysia. The weed plants were washed, air dried ( $27 \pm 3^\circ\text{C}$ ) for 72 h and the whole plant ground by a commercial blender and stored at  $4^\circ\text{C}$  until the time of use. Rice seeds of five varieties namely, MR211, MRQ74, MR220, MR84 and MR232 were obtained from the MARDI Research Station at Seberang Perai, Malaysia.

### Aqueous extract preparation

Ten g of air dried plant parts were placed separately in flasks containing 200 ml of distilled water and shaken for 48 h at room temperature ( $27 \pm 3^\circ\text{C}$ ) on an orbital shaker (160 rpm). The extracts were strained through 4 layers of cheese cloth



**fig2.** Phytotoxic effect of *F. miliacea* debris on the leaf chlorophyll content of 5 rice varieties (MR211, MRQ74, MR220, MR84 and MR232).

and then through two layers of Whatman No-2 filter paper to remove solid materials. The filtrate was centrifuged at 4000 rpm for 15 min. The supernatant was collected and filtered through a 0.22  $\mu\text{m}$  membrane filter paper. The stock solution was stored at 4°C until further use. Four concentrations of the aqueous extracts were used in the experiment i.e: control (0  $\text{g l}^{-1}$ ), quarter strength (12.5  $\text{g l}^{-1}$ ), half strength (25  $\text{g l}^{-1}$ ) and full strength (50  $\text{g l}^{-1}$ ). Dilutions were made prior to use, with distilled water.

#### ***Rice seedlings prepared for MDA content, POD and CAT activity***

Rice seeds were surface sterilized (0.5% sodium hypochlorite for 15 min) and seeds of each variety were placed separately in sterilized large Petri dishes lined with filter paper. The covered Petri dishes were incubated at room temperature (27±3°C). The Petri dishes were checked daily and moistened with distilled water. At 5 days after sowing the newly germinated rice seedlings were transferred to another tray. Then rice seedlings of each variety were treated with the stipulated amount of weed extract for each concentration. Distilled water was substituted in a separate tray for the control. After 3 days of treatment the rice leaves were sampled randomly for the determination of Melondialdehyde (MDA), CAT and POD activity.

#### ***Determination of the MDA content***

The level of lipid peroxidation in the plant tissues was measured by determination of MDA (Liu et al., 2009). The MDA content was determined by the thiobarbituric acid (TBA) reaction. A 0.3 g leaf sample of rice seedling was homogenized with a mortar and pestle in 5 mL 0.1% TCA (Trichloro acetic acid). The homogenate was centrifuged at 10,000×g for 10 min. Four mL of 20% TCA containing 0.5% TBA was added to 1 ml aliquot of the supernatant. The mixture was heated at 95°C for 15 min and cooled immediately. Absorbance was read using the double beam Spectrophotometer (Hitachi U-2000) at 450, 532 and 600 nm after being centrifuged at 10,000×g for 10 min again. The MDA content was calculated using the following formula according to Guo et al. (2006):

$$MDA (\mu\text{M}) = [6.45 (OD532 - OD600) - 0.56 OD450].$$

#### ***Determination of Peroxidase (POD) Activity***

Peroxidase activity was determined using the guaiacol test (Plewa et al., 1991). Three days after treatment of the rice seedlings with the weed extracts, a 0.3 g leaf sample was homogenized in 5 mL of cold (4°C) 50 mM phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate was rapidly filtered through cheesecloth and centrifuged at 11,000 x g for 15 min at 4 °C. The supernatant was taken as the source of peroxidase. The tetraguaiacol formed in the reaction mixture was read using the double beam Spectrophotometer (Hitachi U-2000) at 470 nm. The enzyme was assayed in a solution containing 50 mM phosphate buffer (pH 7.0), 0.3% H<sub>2</sub>O<sub>2</sub> and 1% guaiacol. The reaction was initiated by the addition of 20  $\mu\text{L}$  of the enzyme extract at 25°C. One enzyme unit was calculated on the basis of the formation of 1mM tetraguaiacol for 1 min.

### Determination of catalase (CAT) activity

Catalase (CAT, EC 1.11.1.6) activity was assayed spectrophotometrically by monitoring the decrease in the absorbance of H<sub>2</sub>O<sub>2</sub> at 240 nm using the method of Dhindsa et al. (1981). The assay solution contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H<sub>2</sub>O<sub>2</sub>. The reaction was initiated by the addition of 100 µL of the enzyme extract to the reaction mixture and the change in absorbance was recorded 1 min after the start of the reaction. One unit of activity was considered as the amount of enzyme that decomposes 1 mM of H<sub>2</sub>O<sub>2</sub> in one minute.

### Determination of chlorophyll content

*Fimbristylis miliacea* plant debris at three concentrations (5, 10 and 20 g dry tissue) were mixed together with 1000 g soil separately and placed into black plastic bags (height 12 cm x diameter 10 cm) punched with holes at the bottom and the sides. For the control, similar bags were filled with soil without any plant tissue. Rice seeds were surface sterilized (15% sodium hypochlorite for 15 min) and twenty seeds of each rice variety were sown separately in the bags just below the soil surface and watered regularly at alternate days. Fully expanded rice leaves were collected randomly 2 weeks after sowing. The fresh leaves were cut into small pieces whereby 100 mg were placed in a test tube containing 10 mL acetone (80%) and then kept in a cool room (4°C) overnight. The mixture was strained through glass wool. After centrifugation (5000 rpm) for 10 min at room temperature (25°C), the supernatant was withdrawn and absorbance was recorded at 663 and 645 nm using the Hitachi U-2000 double beam Spectrophotometer. The amount of chlorophyll extracted was calculated using the following formula:

$$\text{Total chlorophyll (g} \cdot \text{l}^{-1}\text{)} = 0.0202 A_{663} + 0.00802 A_{645} \text{ (Arnon, 1949).}$$

### Determination of the mitotic index of onion root

Healthy and equal-sized bulbs of *Allium cepa* L. (onion) were used for the mitotic cell division experiment. After the outer scales were removed and basal ends cut, the onion bulbs were placed in containers, with their basal ends dipping in distilled water and left to sprout under standard laboratory conditions. When the newly emerged roots reached 1.50-2.00 cm in length, they were used for the test. The newly emerged roots were treated with three concentrations of the aqueous extract of *F. miliacea* (12.5 g l<sup>-1</sup>, 25 g l<sup>-1</sup> and 50 g l<sup>-1</sup>). Distilled water was used for the control. After exposure to the extract for 24 h, root tips were cut and subsequently mixed in a freshly prepared mixture of absolute alcohol (ethanol) and acetic acid in the ratio of 3:1 (v/v) for 30 min, washed with distilled water three times, and subjected to hydrolysis in an aqueous solution of 1 N hydrochloric acid (HCl) for 10 min. The roots were then dried and their apical portions (approximately 0.5 cm of root tip) were macerated and dyed with acetic-hydrochloric orcein. Four replicates were carried out for each treatment and scoring was determined from 3 roots in each replicate. The mitotic index was calculated as the ratio between dividing cells and examined total cells. The frequency of each mitotic phase was calculated as the percentage in relation to the dividing cells counted in mitosis.

### Statistical analysis

All experiments were conducted using the complete randomized design with 4 replications. The experimental data

was subjected to the analysis of variance and means were compared using the Duncan Multiple Range Test. Different letters indicate statistical difference at  $p < 0.05$ . The statistical analysis was done using the SPSS/PC version 11.5 software. Figures were plotted by Excel software 2003 (Microsoft Inc., Redmond, Washington, USA). Correlation coefficient and regression analysis were done using Microsoft Excel program.

### Conclusion

The results of this study showed that mixed dried debris of *F. miliacea* in soil exhibited allelopathic effects on the chlorophyll content of rice plant leaves in the green house. The aqueous extract of *F. miliacea* exerted phytotoxic inhibition on the melondialdehyde (MDA) content, the activity of antioxidant enzymes namely catalase (CAT) and peroxidase (POD) of the tested rice varieties. It also caused abnormal mitotic index of the onion root tip. It can be concluded that *F. miliacea*, a common weed in the rice field has allelopathic effect on the cyto-physiological activities of the rice plant. However, more work needs to be done in order to discover whether there are other allelopathic effects that affect other physiological and biochemical parameters of the target species.

### Acknowledgement

This study was supported by research grant No. ERGS/1/2013/STG03/UKM/01/1 (STWN) from the Ministry of Education Malaysia.

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