Key Laboratory of Biology and Genetic Improvement of Maize for Southwest China[1], Ministry of Agriculture, Maize Research Institute, Sichuan Agricultural University, Ya'an; School of Foreign Languages[2], Southwest University Yucai College, Chongqing Hechuan; College of Life and Basic Sciences[3], Sichuan Agricultural University, Sichuan Yaan, PR China

Development of a difenoconazole/propiconazole microemulsion and its antifungal activities against *Rhizoctonia solani* AG1-IA

Pengfei Leng[1,*], Zhiming Zhang[1,*], Qian Li[2], Yunsong Zhang[3], Maojun Zhao[3], Guangtang Pan[1]

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Prof. Maojun Zhao, College of Life and Basic Sciences, Sichuan Agricultural University, Sichuan Ya'an 625014, P. R. China;
zmjun01@yahoo.com.cn
Prof. Guangtang Pan, Key Laboratory of Biology and Genetic Improvement of Maize for Southwest China, Ministry of Agricultural; Maize Research Institute, Sichuan Agricultural University, Ya'an 625014, P. R. China
pangt1956@yahoo.com.cn
* *These authors contributed equally to this work.*

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According to its physical and chemical properties, the composition of difenoconazole/propiconazole microemulsion was as follows: xylene as solvent, emulsifier HSH as surfactant and methanol as cosurfactant. The optimal formulation of difenoconazole/propiconazole microemulsion was oil/SAA/water = 1/2/5 (w/w), in which the SAA consisted of emulsifier HSH and methanol with ratio of 3/2 (w/w). The cloud point of difenoconazole/propiconazole microemulsion was 70 °C and its effective ingredient content was 2.5% measured by High Performance Liquid Chromatography (HPLC). Its heat storage stability was studied according to the standards. The decomposition rates of the difenoconazole/propiconazole microemulsion were merely 2.45%, 2.63% respectively and met the Food and Agriculture Organization (FAO) standards of pesticide microemulsion. Investigated by Transmission Electron Microscopy (TEM) the particle size of difenoconazole/propiconazole microemulsion was 90∼140 nm and its antifungal activities against *Rhizoctonia solani* AG1-IA were tested and compared with that of Meiyu. We found that the inhibition rates in the difenoconazole/propiconazole microemulsion treatment group were significantly higher than that of the emulsion group with the same content of effective ingredients and the study also revealed that its inhibiting ability on the formation and germination of sclerotia was significant.

1. Introduction

Rhizoctonia solani is an important fungal pathogen (Baker 1970) which causes many diseases of host crops and vegetables such as stem canker and black scurf of potato, maize sheath blight, and rice sheath blight and so on. Most scholars believe that the main pathogen of maize sheath blight is *R. solani* AG1-IA, which also could have pathogenicity on rice, wheat and other crops (Marshall and Rush 1980). However, the pathogenicity on maize is especially the strongest (Otten et al. 1999). Over years, chemical pesticides have made a great contribution to the fight against *Rhizoctonia* diseases, but its wide-range use has led to a serious pollution and a dramatic increase of harmful residues in agricultural products. The use of synthetic organic insecticides in crop pest control programs around the world had resulted in damage to the environment, pest resurgence, pest resistance to fungicides, and lethal effects on non-target organisms (Abudulai et al. 2001). So it is urgent to improve the formulation of this traditional fungicide. Biological control of *Rhizoctonia* disease has been demonstrated in some cases (Marin and Robert 2005) and provided effective and sustainable management but still has limitations in practice. Current chemical controls are not completely effective and *Rhizoctonia* disease remains a persistent problem. So new pesticide formulations which are of high efficiency and low environmental pollution are urgent to be developed, microemulsion is such a kind of formulation.

Microemulsions are defined as one transparent, thermodynamically stable homogeneous in macroscale and micro heterogeneous in nanoscale dispersions (Kumar and Mittal 1999). By use of surfactant solubilization, the solvents of liquid or solid pesticides are dispersed in water homogeneously then form a system of optical dispersion transparent or translucent. Microemulsion has lots of advantages compared with other formulations, such as small particle size, stability, high efficiency, low environmental pollution and so on. The United States, Japan, India and France have done research on microemulsion since the 1970 s, involving pesticides, fungicides, herbicides and other fields (Rochling and Albrecht 1993). Since the first report of microemulsion by Schulman, the theory and application of microemulsions has obtained a rapid development since the 1990 s (Tan et al. 2011). A number of articles and reviews on application and theory of microemulsion have been reported (Mittal and Lindman 1984; Mittal 1977).

Under the condition of the same dosage of pesticide effective ingredients, the results showed that laboratory toxicity and field efficacy of microemulsion is superior to emulsifiable concentrate

(EC) and wettable powder (WP), the persistence is longer than EC and cost reduced significantly (Wu and Chen 2009). Li et al. (2009) have developed a 12% triazophos microemulsion which is used for indoor toxicity and field effectiveness experiments, the results show that there is no significant difference between the two formulations. Abamectin microemulsion (0.3%) has been developed and its control effect is similar to 0.5% abamectin EC while greatly reduced the production cost (Li and Li 1999). At present there are many reports on the development and control efficiencies of microemulsion. The effects of property and content of surfactant, cosurfactant and other components have been discussed. Yaghmur et al. (2002) have discussed the microemulsion formulations by addition of short-chain alcohols. It is reported that the addition of short-chain alcohol (such as methanol, ethanol), which increases the penetration of the surfactant film and decreased the polarity of water, seems to favor the formation of microemulsion. Microemulsion preparation, testing and microstructure characterization methods have also been reported in detail (Wang et al. 2007; Al-Adham et al. 2003). Difenoconazole explored by Ciba-Geigy AG. in 1989 is an absorption of triazole fungicides, broad fungicidal spectrum, foliar treatment or seed treatment could improve crops yield and quality (Allen et al. 2004). Propiconazole is a kind of protective and therapeutic adsorption triazole fungicide, which could be absorbed by root, stem, leaf and transmission in plant quickly (Abdul et al. 2008). They have a better control effect on plant disease, such as wheat root rot, powdery mildew, rusts, rice bakanae, sheath blight, maize sheath blight and so on (Gopinath et al. 2006). The microemulsion with the two fungicides can extend the bacterial spectrum and reduce costs with good market potential and broad prospect. The product with difenoconazole and propiconazole as effective ingredients, such as Aimiao, Meiyu, have been available and have better control effect on rice sheath blight (Han et al. 2007). In the present work, attempts are taken to establish a pharmaceutical microemulsion system with xylene as solvent, emulsifier HSH as surfactant, methanol as cosurfactant. And evaluate the physiochemical and other stabilities of the microemulsion. Its antifungal effects against *R. solani* AG1-IA are detected through the method of drug dish in lab. In order to provide laboratory basis for controlling *Rhizoctonia* diseases.

2. Investigations and results

2.1. Preparation of microemulsions

The behavior of multi-components microemulsion was described in a pseudo-ternary phase diagram which was constructed at room temperature as reported recently. The influence of the proportions of methanol in the microemulsion system was studied. Based on the character of surfactant and cosurfactant, the compositions of SAA were designed as HSH/methanol = 2/3, 1/1, 3/2. The pseudo-ternary phase diagrams of the system difenoconazole/propiconazole/methanol/HSH/water were presented in Fig. 1a–c.

As shown in Fig. 1, the microemulsion region increased with the decrease of methanol content. A smaller microemulsion region (87.06%) was formed when HSH/methanol = 2/3 was used (Fig. 1a). with the weight ratio of methanol to HSH raised to 1/1 (Fig. 1b), the one phase region increased (89.50%). When the weight ratio of HSH/methanol was 3/2 (Fig. 1c), a larger one-phase region dominated most of the phase diagram area (92.20%). As the variation was small, the phases diagram constructed by higher methanol content of SAA was not studied. Comparing Fig. 1a–c, Fig. 1c was chosen for formula screening as its maximum region of the microemulsion area. Several candidates which consist of certain contents of oil, SAA and

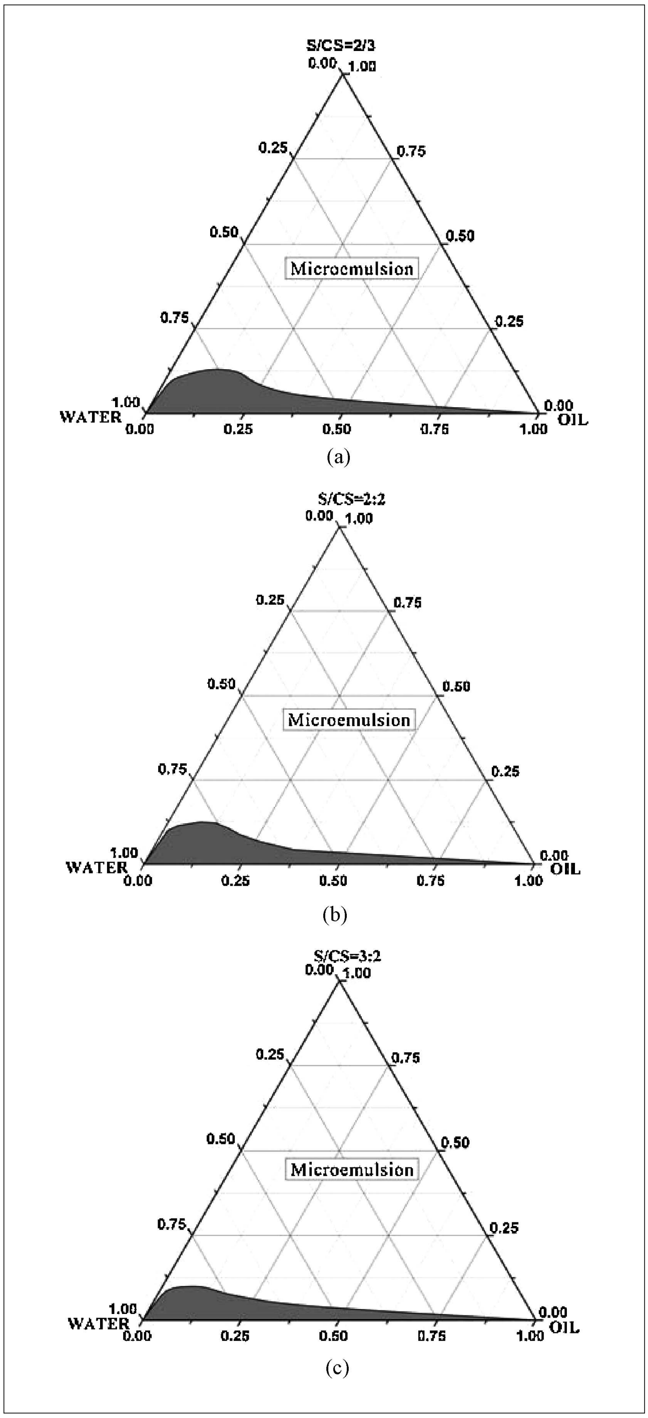

Fig. 1: Pseudo-ternary phase diagrams of the system difenoconazole/propiconazole/methanol/HSH/water at 25 °C with different ratio of HSH/methanol: (a) HSH/methanol (2/3), (b) HSH/methanol (1/1), (c) HSH/methanol (3/2)

water were selected. After testing parts of their properties, the best candidate of microemulsion was determined as follows: Oil/SAA/water = 1/2/5, SAA consists of HSH and methanol with ratio of 3/2. The microemulsion prepared according to the above formula was further tested.

2.2. Results of physiochemical detection

2.2.1. Type of difenoconazole/propiconazole microemulsion

The appearance of the microemulsion was in good liquidity, with light blue fluorescence and no turbidity or crystallization, which

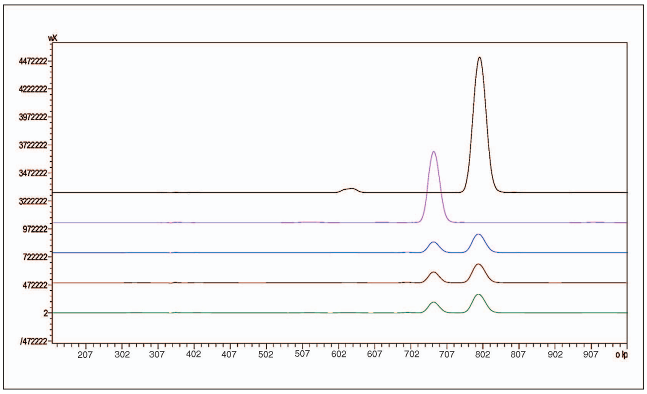

Fig. 2: The chromatogram of standard difenoconazole/propiconazole solution and microemulsion

were typical characteristics of a microemulsion. In view of the colloidal properties of the microemulsion, combined with the Tyndall effect its appearance was tested in this study. A beam of light could be seen clearly after a monochromatic light on the sample. No phase separation was observed in the sample after centrifuging at 12 000 rpm for 20 min at room temperature. The diffusion velocity of methylene blue solution in the microemulsion sample was significantly faster than Sudan in which there was no obvious diffusion, and diffusion velocity of the sample in distilled water was also faster than that in mineral oil. All this proved that the type of the sample was O/W.

2.2.2. Stability of difenoconazole/propiconazole microemulsion

The contents of effective ingredients were analyzed by high performance liquid chromatography. The experiments were performed in triplicate under the same conditions. The chromatogram is shown in Fig. 2, retention times of propiconazole and difenoconazole were 5.31 min and 5.95 min, respectively. At 230 nm, good peak symmetry was formed; the impurity response value was low and no unidentified peaks on the main peak in the HPLC chromatograms and the effective ingredients and impurities could be completely separated. The contents of three parallel tests of difenoconazole and propiconazole were 2.50%, 2.54%, 2.51% and 2.71%, 2.72%, and 2.68%, respectively. The results showed that the standard deviations and variation coefficients of difenoconazole and propiconazole were 0.01, 0.58% and 0.02, 0.96%, respectively. The average concentration of difenoconazole and propiconazole was 2.52% and 2.70%. As can be seen from Fig. 2, the method precision was better and was considered to be adequate for analytical assays. The effective ingredients content of the microemulsion sample was 2.5%. Parts of the physiochemical stability test results were as follows.

Dilution stability: Difenoconazole/propiconazole microemulsion was diluted with standard hard water (content of Ca^{2+}, Mg^{2+} were 342 mg/L) for 20, 50, 100, 200 times. Before and after water bath each sample maintained a transparent uniform, no floating oil and sediment.

Low temperature stability: After having been placed at 0 °C for 14 days, there was no crystallization, delamination, no significant change in mobility and emulsifying properties.

Heat storage stability: After having been stored at 54 °C for 14 days, there was no cloud and delamination, the content of difenoconazole and propiconazole in the sample was 2.45% and 2.63%, respectively, and the decomposition rate was less than 5%, which met the requirement of FAO. It was suggested that the microemulsion could be used in high-temperature area.

Freezing experiment: Frozen at −18 °C, the microemulsion could still be restituted at room temperature. There was no change in mobility and emulsifying capacity, which was in accordance with the reported testing results of pesticide microemulsion.

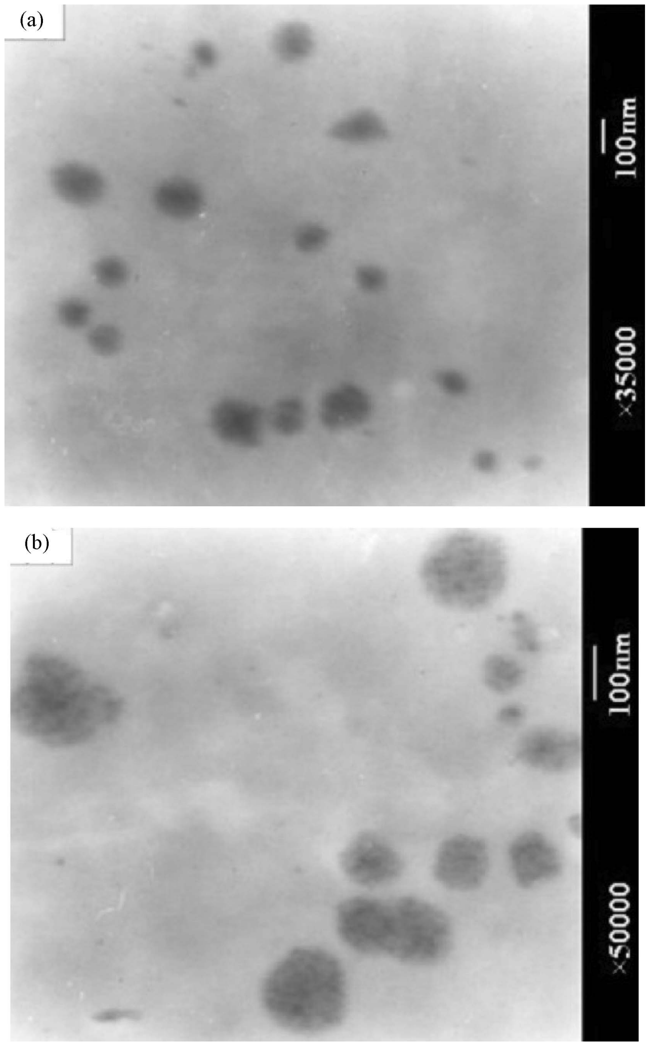

Fig. 3: Transmission electron microscopic photomicrographs of microemulsion: (a) 10 times dilution, (b) 50 times dilution

Cloud point: The cloud point of the difenoconazole/propiconazole microemulsion sample was 70 °C after detection.

In this study, samples with distilled water, water, and standard hard water were prepared following the same formula, and parts of physiochemical test results are shown in the Table. The stability of the system varies with the increase of Ca^{2+}, Mg^{2+} especially the cloud point. There was no obvious dissimilarity in appearance among the three samples, which were in good liquidity with a light blue appearance. A Tyndall effect could also be detected, prepared with different water quality. The maximum difference of cloud point was 10 °C, which indicated that water quality had a strong effect on the sample (from 60 °C to 70 °C), but it still could meet the requirement for heat storage. But in the low temperature and freezing experiments, the sample prepared with standard hard water delaminated and could be restituted at room temperature. In view of the above results, it was highly recommended that this product should not be prepared with harder water than that used here.

2.3. Particle size measurement

As shown in Fig. 3a, the particles were circular and well-distributed, which was in line with the typical microscopic

Table: Effect of different water quality on stability of difenoconazole/propiconazole microemulsion

Detection index	Water quality		
	Distilled water	Water	Standard hard water
Appearance	Light blue, transparent, Tyndall effect	Light blue, transparent, Tyndall effect	Light blue, transparent, Tyndall effect
Cloud point (°C)	70	64	60
Low temperature stability	Clear, transparent	Clear, transparent	Slightly stratified, Restitution at room temperature
Freezing experiment	Frozen, Restitution at room temperature	Frozen, Restitution at room temperature	Slightly stratified, Restitution at room temperature

structure of microemulsion. By calculating the diameter of difenoconazole/propiconazole microemulsion was 90∼140 nm. The particles remained stable but their diameter was becoming smaller after being diluted for 50 times (see Fig. 3b).

2.4. *Antifungal activity assay*

2.4.1. *Inhibition on hypha growth*

Under different stages of treatment time, the inhibition ability of difenoconazole/propiconazole microemulsion and Meiyu with different concentrations on *R. solani* hypha growth was compared as shown in Fig. 4a–d. As can be seen from Fig. 4, with the raise of pesticide concentration, the growth of *R. solani* AG1-IA was gradually inhibited under the two treatment conditions and showed a better growth rule. When the concentration of difenoconazole/propiconazole microemulsion was 0.25 mL/L, the inhibitory rate remained above 92% (see Fig. 4a). At any processing conditions, the inhibitory rate of microemulsion was higher than that of Meiyu. When the concentration of the microemulsion was 1 mL/L, the concentration of difenoconazole/propiconazole was almost 0.06 g/L, the inhibitory rate could reach 100% (see Fig. 4c) while 0.4 mL/L of Meiyu, the concentration difenoconazole/propiconazole was almost 0.12 g/L (see Fig. 4d) could achieve this effect. When the concentration of difenoconazole/propiconazole microemulsion

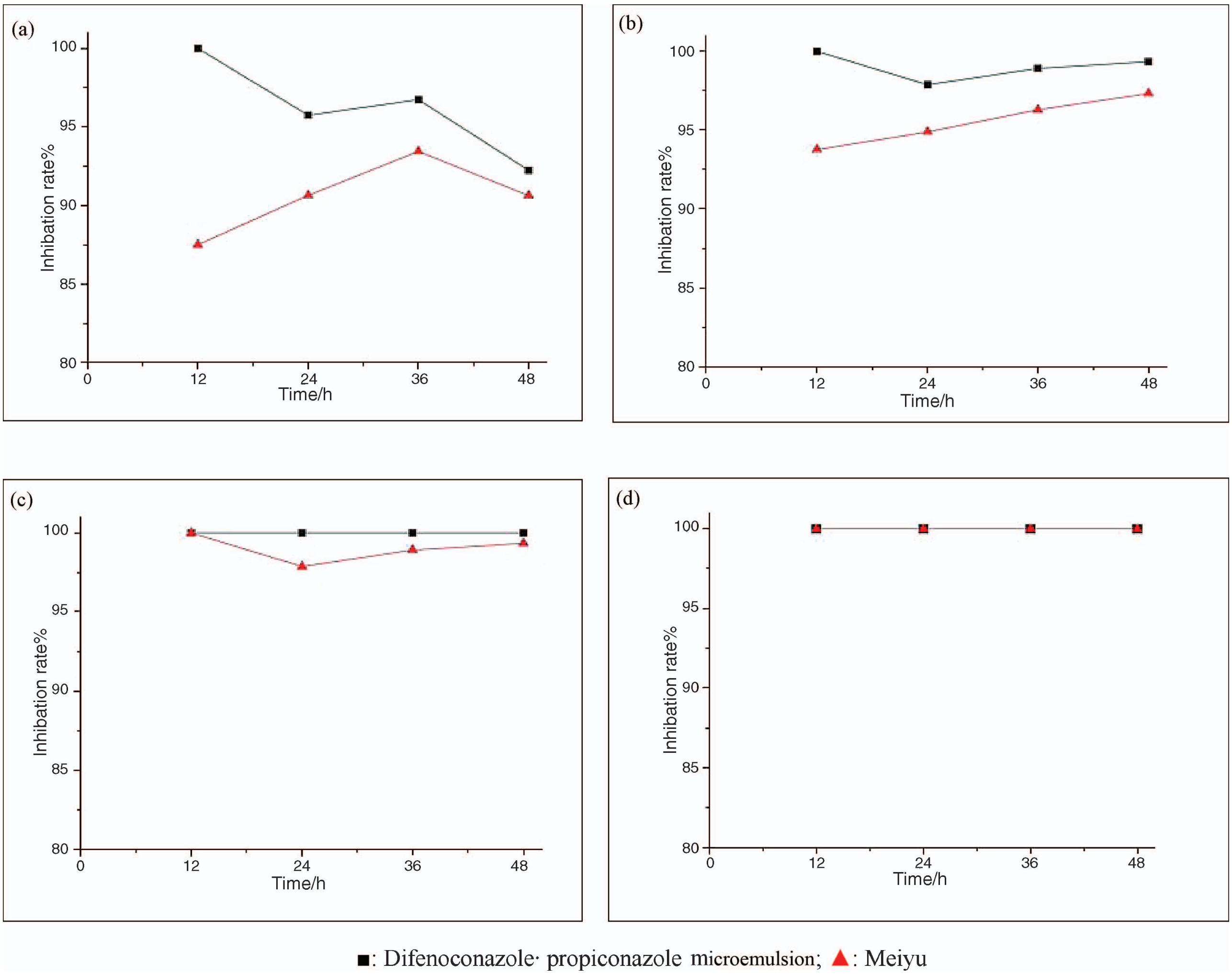

Fig. 4: The inhibition ratio contrast between difenoconazole/propiconazole microemulsion and Meiyu: (a) concentration of microemulsion and Meiyu were 0.25 mL/L and 0.05 mL/L, (b) concentration of microemulsion and Meiyu were 0.5 mL/L and 0.1 mL/L, (c) concentration of microemulsion and Meiyu were 1.0 mL/L and 0.2 mL/L, (d) concentration of microemulsion and Meiyu were 2 mL/L and 0.4 mL/L

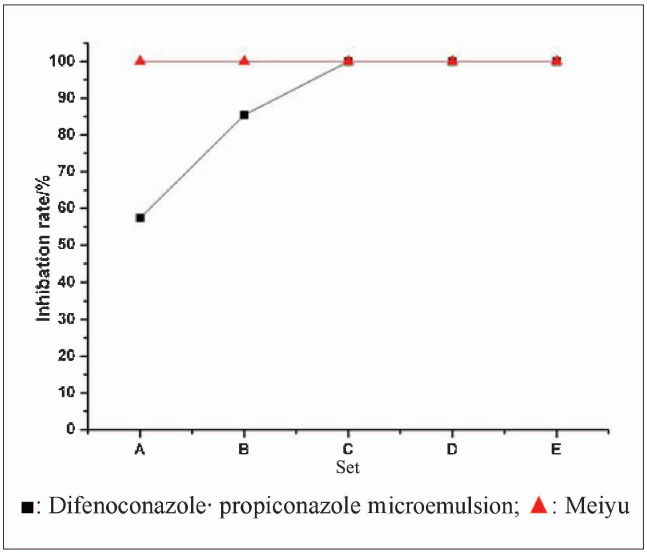

Fig. 5: The inhibition on formation of *R. solani* AG1-IA sclerotia

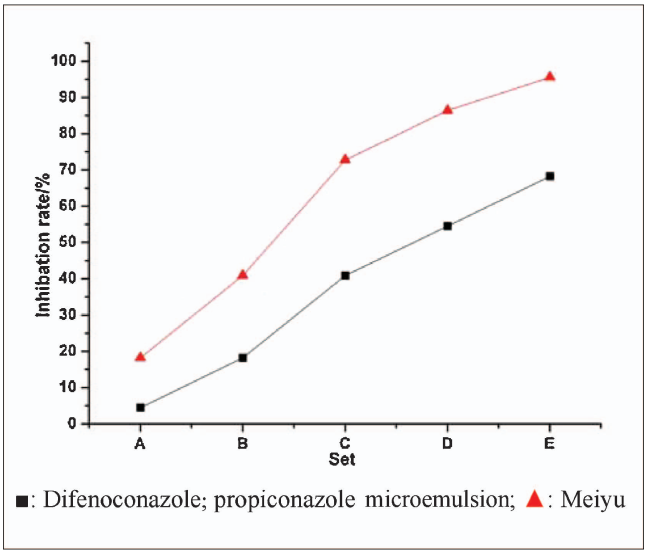

Fig. 6: The inhibition on fgermination of R, solani AGI-IA sclerotia

was 2 mL/L and the concentration of Meiyu was 0.4 mL/L, the hypha growth of *R. solani* both were completely inhibited (see Fig. 4d). As the inhibitory rate of higher concentration was the same with Fig. 4d and was not shown in this paper. The above results indicated that difenoconazole/propiconazole microemulsion had a better inhibiting effect on hypha growth of *R. solani* AG1-IA than Meiyu with the same active ingredient content.

2.4.2. *Inhibition on sclerotia formation*

The dry weight of sclerotia results showed that the formation of *R. solani* AG1-IA sclerotia was completely inhibited by Meiyu within 15 days' cultivation. The inhibitory effect of difenoconazole/propiconazole microemulsion was enhanced with the increase of concentration (shown in Fig. 5). The inhibition of Meiyu was higher than the microemulsion when the concentration was under 1 mL/L throughout the cultivation. And a complete inhibition occurred when the concentrations of microemulsion and Meiyu were above 1 mL/L, which was related to the results of the inhibition on *R. solani* AG1-IA hypha growth.

2.4.3. *Inhibition on sclerotia germination*

The degree of mature sclerotia germination was different after treating in different concentrations of difenoconazole/propiconazole microemulsion and Meiyu. As shown in Fig. 6, when the sclerotia were cultivated for 24 h, with the increase of concentration, the inhibition on sclerotia germination efficiency was increasing correspondingly and so was their difference value. It can also be observed that the inhibition effect of Meiyu was always better than that of the microemulsion.

3. Discussion

Methanol, as co-surfactant, plays an important role in reducing interfacial tension and adjusting the Hydrophilic-Lipophilic Balance value (HLB). A microemulsion is easily formed because small methanol molecules are easier to insert into surfactant, thereby lower the interfacial film rigidity, increase its mobility and reduce the bending energy which microemulsion needs when it comes into formation. Fig. 1 shows that the microemulsion region is increasing slightly from 87.06% to 92.20% as the methanol content is diminishing, but the difference is not significant. It can be predicted that the microemulsion region would keep increasing slightly to a certain degree with the continuing decrease of methanol proportion but the change might not be obvious. Therefore, we do not do other research in our work to provide data basis for this, although the increase of methanol content is significant in improving the appearance and other stabilities of microemulsion system. The fact that the microemulsion region did not expand in accordance with the rise of methanol content indicates that a higher content of methanol is of limited benefit. A threshold exists, above which the microemulsion region begins to shrink even though the declination is not very obvious. With the increase in methanol proportion, notable improved microemulsion liquidity and appearance leading to a more stable and uniform formulation. One possible explanation is that the addition of methanol enhances oil incorporation into the interface, due to the hydroxyl group in HSH molecular structure, which makes it well-dissolved in methanol.

Pesticide microemulsion is a water-based system mostly belonging to the oil-in-water (O/W) type which contributes a lot to environment improvement. The diffusion velocity of microemulsion is closely related to its characteristic. As its continuous phase is water, the O/W type is strongly hydrophilic and water-in-oil (W/O) microemulsion is the opposite. The diffusion velocity of O/W microemulsion in water is very fast while there is no diffusion in non-polar mineral oil and the diffusion velocity of water-soluble methylene blue in O/W microemulsion is much faster than in oil-soluble Sudan. These results are consistent with a previous report by Fang et al. (2009) and all of the above could be explained by the theory that solute and solvent with similar properties are mutually soluble. In addition, the ratios of components have great impact on the type of microemulsion, the more content of methanol, water and other polar material the closer it is to O/W. In this study, methanol 10%, water 62.5% and amphipathy surfactant HSH 15%, the content of these also determined the type of the sample is O/W.

The cloud point is an important index for the physical stability of microemulsion. The polyoxyethylene oxygen atom of nonionic emulsifiers are linked with water through hydrogen bonds, which are sensitive to temperature and break when the temperature reaches a certain degree, leading to a decrease of surfactant hydrophilicity and HLB, and eventually result in phase separation, due to which the microemulsion system will turn from transparent to opaque. Besides, HLB value of surfactant, content of Ca^{2+}, Mg^{2+}, carbon chain length of cosurfactant and other factors could influence the cloud point of a microemulsion. From the results of this research we can see that with an increase in HLB, the cloud point of the microemulsion increased. But with increasing concentration of Ca^{2+}, Mg^{2+} and increasing length of alkyl chain of cosurfactant, the cloud point of the microemulsion decreased. The above conclusions are in accordance with the research of Song et al. (2005).

Water quality also has various impacts on the physiochemical properties of a microemulsion. Influenced by the water quality, the efficacy of the pesticide microemulsion in different areas varies a lot. So it is necessary to study the influence of water quality in processing and application. Microemulsion systems containing a ionic surfactant are sensitive to electrolytes, so phase transition will occur as electrolyte concentration changes. The concentration of Ca^{2+}, Mg^{2+} in water (water hardness) is an important factor for the stability of microemulsions. This paper studied the impact of water hardness on the appearance, steadiness at low temperature, freeze resistance and cloud point of the microemulsion. As shown in the Table, along with the increase of water hardness the cloud point decreased and the stability, accordingly, should be reduced as suggested in other studies (Kabalnov et al. 1995; Chen et al. 2000). We infer that the related reasons may be as follows: as the water hardness is increasing, the concentration of Ca^{2+}, Mg^{2+} is also rising, which results in the decrease of surfactant HLB value and a series of changes in the microemulsion system including the interface film becoming thinner, the oil droplet larger and more uneven, and the particles are prone to collide with each other leading to phase separation especially when temperature is increased. All these reasons cause instability of a microemulsion system and a decline of cloud point.

Al-Navajeh (2003) has analyzed the relationship between antimicrobial activity and the position of the microemulsion within its existence area (or stability region), and suggested that the maximal activity is found at the centre of the stability region. As shown in Fig. 4a–d, the results give clear evidence of a good inhibition activity of the microemulsion against *R. solani*. On the basis of same effective ingredient content, inhibition by the microemulsion is better than that by the emulsion. This may be related to the special physiochemical properties, higher transference efficiency and other advantages of a microemulsion. The main reason may be that pesticide microemulsions could permeate into the tissue easily and thus enhance the efficacy of pesticides. The use of microemulsions achieves two mutual compensation effects: one is that the pesticide can easily overcome the capillary resistance due to the decreased interfacial tension; the other is that the improved solubilization of the pesticidal ingredient increases the gradient between liquid and plant tissue and then promotes the transmission velocity through diffusion. In addition, another possible reason is that with respect to water medium, the existence of surfactant and other additives may have an inhibiting effect on the growth of pathogens.

Sclerotia of *R. solani* are the main source of *Rhizoctonia* diseases, so it is important to inhibit the formation and germination of sclerotia for preventing crops disease. Therefore, we studied the inhibition on formation and germination of sclerotia by the difenoconazole/propiconazole microemulsion to provide a research basis for Rhizoctonia diseases field prevention. Figure 5 indicates that with increasing pesticide concentration the inhibition efficacy on sclerotia formation is gradually enhanced and when the concentration reaches 0.1 mL/L, complete inhibition appears, which is closely related to the inhibition ability of difenoconazole/propiconazole microemulsion on hypha growth. It should be noted that the hypha could still grow and form a number of sclerotia after being cultivated for some time at a lower concentration. It can be observed from Fig. 6 that the sclerotia germination rate declines with increase of system concentration, but the inhibition ratios in difenoconazole/propiconazole microemulsion are lower than that in Meiyu. After treated in microemulsion, the hypha growth is seriously inhibited, thus the mature sclerotia germination ratio is reduced to a large degree. The reason for this is that the microemulsion reduces the interfacial tension and makes it easier for the pesticide particles to overcome the capillary resistance and to penetrate into the tissue. From the suppression process of growth, formation and germination of *R. solani* AG1-IA by difenoconazole/propiconazole microemulsion, it can be found that complete inhibition appeared when the pharmaceutical concentration reached a certain value. Furthermore, it is noteworthy that the suppressive effect on hypha growth and sclerotia formation was better that on the sclerotia germination. These results are closely related to the fungicidal mechanisms of two fungicides. Difenoconazole, belonging to the systemic sterol demethylation inhibitors class, has the efficacies of protection and treatment, which are conducive to the inhibition on the biosynthesis of cell wall and the growth of fungi. Propiconazole is a triazole fungicide, which has the potential to control sterol biosynthesis and damage pathogens cell membrane function, finally resulting in cell death. Thus, the supression of *R. solani* AG1-IA by difenoconazole/propiconazole microemulsion does not mainly rely on sclerotia germination but on hypha growth. This result is in accordance with the report of Wang et al. (2007) in which they claimed that the inhibition ability of tebuconazole is largely related to sclerotia germination rather than on hypha growth. It can be predicted that the microemulsion formulated with difenoconazole and propiconazole will have good suppression effect on a variety of diseases, such as rice sheath blight, rice bakanae, wheat root rot and so on. And we will do further studies on the control of maize sheath blight and rice sheath blight.

4. Experimental

4.1. Materials

4.1.1. Chemicals

Difenoconazole, propiconazole and Meiyu were purchased from Ji-Nan Shibang Agrochemical Co. Ltd. Difenoconazole and propiconazole standard were bought from SIGMA-ALDRICH (Germany). Emulsifier HSH (Jiangsu Hai-an Shi You Chemical Co.) was of commercial grade. Sudan IV and Eosin blue were of analytical grade and supplied by the Sinopharm Group Chemical Reagent Co. Ltd., (Shanghai, China). Methanol was purchased from Sichuan Xilong Chemical Co. Ltd. The water was distilled.

4.1.2. Microorganism

R. solani AG1-IA was obtained from Rice Research Institute, Sichuan Agricultural University (Yaan, China). It was maintained on potato dextrose agar medium (PDA: potato, 200 g; dextrose, 20 g; agar, 20 g; and deionized water, 1000 mL) at 28 °C.

4.2. Construction of pseudo-ternary phase diagrams

In this study, the law of emulsifiable oil was used to develop a difenoconazole/propiconazole microemulsion (Hiroshi et al. 2005). The pseudo-ternary phase diagrams were constructed with data from SAA (mixture of surfactant and co-surfactant) titration measurements. Various ratios (w/w, 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2 and 9/1) of oil and SAA (emulsifier HSH and methanol by ratio of 2/3, mass ratio, the same below) were dispersed in vials. To each vial, water was added dropwise. When a critical change in transparency was observed, the sample was made and the data of water consumption, oil and SAA from titrations of the 9 vials were recorded for later construction of pseudoternary phase diagram by Origin7.5, so did the phase diagrams with HSH and methanol ratios (1/1, 3/2). Compared the size of microemulsion region with different ratios of HSH and methanol, the diagram with maximum microemulsion region was selected as the candidate. Based on an overall consideration of content of pesticide, emulsifier, water and other factors, the optimal formula of difenoconazole/propiconazole microemulsion was determined. Then the microemulsion was prepared according to the formula for further testing.

4.3. Physicochemical stabilities

4.3.1. Study the type of microemulsion

In this research, appearance observation, centrifugation, staining and dilution were used to study the type of microemulsion. First, it was observed, whether the system was stable, the appearance of the sample was transparent and with a slightly light blue fluorescence, and if there was crystallization, turbidity or sedimentation. Secondly, the sample was centrifuged at the speed of 12 000 rpm for 20 min to test whether there was sedimentation or not. Besides, two groups were compared, the diffusion velocity of oil-soluble Sudan and water-soluble methylene in the sample, and the diffusion velocity of microemulsion in distilled water and mineral oil (Zhong et al. 2009).

4.3.2. Stability of microemulsion

The concentrations of difenoconazole/propiconazole were determined by HPLC analysis using a Shimadzu (Kyoto, Japan) liquid chromatography, equipped with LC–10 AT VP solvent pump unit and SPD–10A VP UV–Visible detector. Samples were injected manually through a 5 μL loop with a rheodyne injector. The separation was performed in a C18 VP–ODS, 250 × 4.6 mm column with a mobile phase of acetonitrile/water (70/30 v/v) containing acetic acid with a pH of 3.0 (flow rate of 1.0 mL/min). Difenoconazole and propiconazole were detected at 230 nm according to Xu et al. (2009). Column temperature: room temperature. Under these conditions, the standard sample solutions were injected and each response value was calculated after the equipment was stable. The sample was tested when the peak area variation of two standard solutions was less than 1.5%. All measurements were carried out at room temperature. The content of difenoconazole and propiconazole were calculated by the following formula

$$C = \frac{A_2 M_1 P}{A_1 M_2} \times 100\%$$

where: A_1: peak area of standard sample; A_2: peak area of sample solution; M_1: the quality of standard sample (g); M_2: the quality of the sample (g); P: concentration of standard samples (%).
There are few detailed reports on the quality standards of microemulsions. In view of former research and studies, excellent physiochemical stability microemulsions should be achieved (Aboofazeli et al. 2004). According to Aboofazeli (1995) and Chen (2004), the following five aspects should be evaluated, emulsion stability, low temperature stability, freezing experiment, cloud point, and heat storage stability. The first four aspects were detected for the three kinds of microemulsion samples prepared by distilled water, water, and standard hard water.

4.3.3. Characterization of microemulsion system

Characterization of microstructure also played an important role in the study of the microemulsions. At present there are several methods for microstructure determination, such as NMR, infrared spectroscopy, light scattering, electrical conductivity and other electron microscopy (Hasse and Keipert 1997; Klang et al. 1994). In this study, the transmission electron microscopy (TEM) was chosen to study the microstructure. The microemulsion was diluted 10 and 50 times with ultrapure water. After filtering through a 0.45 μm membrane, the samples were placed on a carbon-coated copper grid and then 1% phosphotungstic acid was dropped to cover the samples. The superfluous phosphotungstic acid on the samples was wiped off using a filter paper. Then they were observed in the transmission electron microscopy of Model H-600A-2 (JEOL, Japan).

4.3.4. Antifungal activities against R. solani AG1-IA

Inhibition activity against *R. solani* AG1-IA was determined by the method of drug dish (Zhang 1999). The mixture of difenoconazole/propiconazole microemulsion and culture medium was prepared with Meiyu (the contents of difenoconazole and propiconazole were 300 g/L) as contrast and a blank control group was also set (Fu et al. 2006). Difenoconazole/propiconazole microemulsion concentrations were 0.25, 0.5, 1, 2, 4 mL/L and Meiyu concentrations were 0.05, 0.1, 0.2, 0.4, 0.8 mL/L, respectively. Then the medium were inoculated with *R. solani* AG1-IA (diameter was 0.4 cm) which was active and growing well (diameter was 0.4 cm). While being cultured in the darkness at 28 °C, the diameters were measured by cross method and inhibition rate was calculated (see formulae) every 12 h. The experiments were performed in triplicate for each set of conditions.

$$\text{Inhibition} = \frac{D_0 - D_1}{D_0} \times 100\%$$

where: D_0: blank control diameter; D_1: set diameter.
The PDA plates with the concentration of difenoconazole/propiconazole microemulsion (0.25, 0.5, 1, 2, 4 mL/L) compared with Meiyu (0.05, 0.1, 0.2, 0.4, 0.8 mL/L) recorded as A, B, C, D and E, were inoculated with *R. solani* AG1-IA. After cultivating for 15 days at 28 °C, sclerotia was picked out and dried to constant weight at 80 °C. And the inhibition ratio on sclerotia formation by difenoconazole/propiconazole microemulsion was calculated. The experiments were performed in triplicate for each set.
The matured sclerotia of *R. solani* AG1-IA were dipped in liquid with difenoconazole/propiconazole microemulsion (0.25, 0.5, 1, 2, 4 mL/L) compared with Meiyu (0.05, 0.1, 0.2, 0.4, 0.8 mL/L) recorded as A, B, C, D and E for 30 s and then air dried. That one dipped in sterile water was selected as blank control group. The treated sclerotia was put in PDA plate and cultivated at 28 °C for 24 h. The experiments were performed in triplicate for each set which consisted of 10 sclerotia.

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