USING HANDS-ON YEAST BIOLOGICAL CONTROL FOR *COLLETOTRICHUM CAPSICI* TO TEACH ORGANISM INTERRELATIONSHIP CONCEPTS AND TO ENCOURAGE CRITICAL THINKING

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Thesis

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Arun Chanchaichaovivat

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ABSTRACT

Yeasts antagonistic to *Colletotrichum capsici* were isolated from 11 species of Thai fruits and vegetables. Four antagonists (R13, R6, ER1, and L2) were found to inhibit *C. capsici* growth with biological control efficacies of 93.3%, 83.1%, 76.6%, and 66.4%, respectively. They were identified by using 26S rDNA and ITS region sequences together with physiological and morphological characteristics, and found to be *Pichia guilliermondii*, *Candida musae*, *Issatchenkia orientalis*, and *Candida quercitrusa*. The most effective strain, *P. guilliermondii* R13 reduced disease incidence in *C. capsici* infected chilli fruits to as low as 6.5%, and was more effective in preserving chilli fruits than conventional preservation with chlorinated water. The modes of action of *P. guilliermondii* strain R13 against *C. capsici* was examined both on agar plates and in chilli wounds. Light microscopy indicated that strain R13 attached to the fungal hyphae and that attachment was associated with restricted proliferation of *C. capsici* in chilli fruit wounds. Studies on competition for nutrients revealed that the suppression of spore germination and germ tube length of *C. capsici* by *P. guilliermondii* strain R13 was significantly diminished by glucose, sucrose, and some nitrate sources. Strain 13 also produced hydrolytic enzymes, including β-1,3-glucanase and chitinase, in both solid and liquid media. These enzymes showed highest activities when the *C. capsici* hyphal cell wall, rather than laminarin or glucose, was the sole carbon source. Unlike other strains tested, strain 13 did not produce a killer toxin when cultivated under similar conditions. Multiple modes of action may make strain R13 a good candidate for biological control of chilli anthracnose.

Results from this study were used to design hands-on biological control activities for 11th grade students based on the experiential learning model. Students in the experiential learning class showed higher conceptual understanding of organism interactions in biological control systems and were more critical about their roles in the environment compared to the traditional learning class. They were assessed for their abilities to give better explanations in open-ended questions and interviews, and to construct a more complex concept map. The students also applied their knowledge to design science projects, which were evaluated as ‘superior’ using the Rubric score. They had positive attitudes towards this hands-on activity, not only in their learning outcomes, but also in their environmental awareness. This advanced instructional method, using yeast biological control experiment, enhanced students’ conceptual understanding and critical thinking skills. It could thus be used as an alternative teaching method to improve learning outcomes of students studying Biology.

KEY WORDS: BIOLOGICAL CONTROL/ YEAST ANTAGONISTS/ EXPERIENTIAL LEARNING/ CRITICAL THINKING/ CONCEPTUAL UNDERSTANDING

180 P.

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การใช้บทปฏิบัติการเรื่องการควบคุมรา *COLLETOTRICHUM CAPSICI* โดยยีสต์ในการสอนมโนทัศน์และความคิดเชิงวิพากษ์ของความสัมพันธ์ระหว่างสิ่งมีชีวิต (USING HANDS-ON YEAST BIOLOGICAL CONTROL FOR *COLLETOTRICHUM CAPSICI* TO TEACH ORGANISM INTERRELATIONSHIP CONCEPTS AND TO ENCOURAGE CRITICAL THINKING)

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บทคัดย่อ

ในการคัดแยกยีสต์ปฏิปักษ์ต่อรา *Colletotrichum capsici* ที่ทำให้เกิดโรคแอนแทรกโนสจากผลไม้และผักของไทย จำนวน 11 ชนิด พบยีสต์ที่มีประสิทธิภาพสูงในการควบคุมการเจริญของรา จำนวน 4 สายพันธุ์ คือ R13 R6 ER1 และ L2 ซึ่งมีประสิทธิภาพควบคุมโรคได้ 93.3% 83.1% 76.6% และ 66.4% จากการบ่งชี้ชนิดของยีสต์เหล่านี้ด้วยวิธีวิเคราะห์ลำดับเบสบนสายดีเอ็นเอบริเวณ 26S rDNA และ ITS พร้อมด้วยลักษณะทางสรีรวิทยา และรูปร่างของเซลล์ พบว่า ยีสต์เหล่านี้ คือ *Pichia guilliermondii Candida musae Issatchenkia orientalis* และ *Candida quercitrusa* ตามลำดับ ซึ่ง *P. guilliermondii* สายพันธุ์ R13 สามารถลดการเกิดโรคในผลพริกให้เหลือเพียง 6.5% และพบว่ามีประสิทธิภาพดีกว่าการใช้น้ำคลอรีนที่มักใช้กันในการเก็บรักษาผลพริกหลังการเก็บเกี่ยว การทดลองนี้ได้ศึกษากลไกที่ยีสต์สายพันธุ์ R13 ลดการเกิดโรคจาก *C. capsici* ทั้งบนจานเพาะเชื้อและบนผลพริก จากการสังเกตด้วยกล้องจุลทรรศน์ พบว่าเซลล์ยีสต์จะไปเกาะเส้นใยราทำให้ลดการเจริญของราบนบาดแผลของผลพริก เมื่อเพิ่มสารอาหารกลูโคส ซูโครส และแหล่งไนเตรทบางชนิดลงในอาหารเลี้ยงเชื้อที่มีน้ำคั้นจากผลพริก พบว่าการงอกและความยาวของสปอร์ราถูกยับยั้ง แสดงว่าการแย่งสารอาหารระหว่างยีสต์สายพันธุ์ R13 และ *C. capsici* เป็นกลไกหนึ่งในการควบคุมโรคของยีสต์ชนิดนี้ นอกจากนี้ยังพบว่ายีสต์สายพันธุ์ R13 สามารถสร้างเอนไซม์ไคติเนสและเบต้า-กลูคาเนสได้ทั้งในอาหารเลี้ยงเชื้อชนิดแข็งและเหลว เอนไซม์ทั้งสองชนิดนี้มีความสามารถในการทำงานสูงสุดในอาหารที่มีแหล่งคาร์บอนเตรียมจากผนังเซลล์ *C. capsici* ซึ่งจะมากกว่าในอาหารที่มีลามินารินและกลูโคส 2 และ 15 เท่า ตามลำดับ จากการทดลองนี้ไม่พบการสร้างสารคิลเลอร์ท็อกซินของยีสต์สายพันธุ์ R13 ในสภาวะที่ใช้เลี้ยง การที่ยีสต์สายพันธุ์ 13 มีกลไกการยับยั้งการเจริญของ *C. capsici* ในหลายลักษณะอาจทำให้มีโอกาสสูงที่จะนำไปใช้ควบควบคุมราก่อโรคแอนแทรกโนสในผลพริก

ผลส่วนหนึ่งจากการทดลองใช้ยีสต์ควบคุมโรคแอนแทรกโนสในผลพริกได้นำมาสร้างเป็นบทปฏิบัติการสำหรับนักเรียนชั้น ม. 5 โดยได้นำมาบูรณาการเข้ากับหลักการเรียนการสอนแบบ experiential learning model หรือ การเรียนรู้จากประสบการณ์จริง ผลการทดลองพบว่ากลุ่มนักเรียนที่เรียนรู้จากประสบการณ์จริงสามารถสร้าง conceptual understanding ในเรื่องความสัมพันธ์ระหว่างสิ่งมีชีวิตจากการทดลองทำปฏิบัติการเรื่องการควบคุมโรคพืชโดยชีววิธีที่พัฒนาขึ้น พร้อมทั้งมีทักษะความคิดเชิงวิพากษ์มากกว่ากลุ่มที่เรียนรู้จากการสอนด้วยวิธีดั้งเดิมแบบการบรรยาย ทั้งนี้ได้ประเมินจากความความสามารถในการตอบและอธิบายปัญหาปลายเปิด การสัมภาษณ์ และความซับซ้อนของแผนผังมโนทัศน์ที่นักเรียนสร้างขึ้น นอกจากนี้กลุ่มนักเรียนที่เรียนรู้จากประสบการณ์จริงยังได้นำความรู้จากการทำปฏิบัติการในชั้นเรียนไปออกแบบโครงงานวิทยาศาสตร์ซึ่งมีผลงานอยู่ในเกณฑ์ดีเยี่ยม นักเรียนมีทัศนคติที่ดีในแง่ที่ว่าการเรียนโดยลงมือทำเองช่วยให้เกิดการเรียนรู้ได้มากขึ้น นอกจากนี้เนื้อหายังส่งเสริมความสำนึกเรื่องสิ่งแวดล้อม การเรียนรู้โดยวิธีดังกล่าวนี้ได้ช่วยส่งเสริมความคิดเชิงวิทยาศาสตร์และความคิดสร้างสรรค์ ซึ่งรูปแบบการสอนนี้เป็นอีกวิธีการหนึ่งที่สามารถนำไปใช้เพื่อส่งเสริมการเรียนรู้ของนักเรียนในวิชาชีววิทยาได้

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CHAPTER I
INTRODUTION

This chapter presents considerable evidences in scientific and educational rationale leading to research design in this study.

Scientific approach

Biological control is the method using one organism including its derivative to control another. The biological control control agents may be antagonistic microorganisms or even natural plant- and animal- derived compounds (Pal and Gardener, 2006). During the past decades, this method was developed to be an alternative way to reduce chemical pesticide contamination on foods and in the environment, and considerable success has been achieved upon utilizing antagonistic microorganisms to control both preharvest and postharvest diseases (Janisiewicz and Korsten, 2002). A variety of microbial antagonists have been reported to control several different pathogens on various fruits and vegetables (Fravel, 2005; Mari and Guizzardi, 1998).

Among the antagonistic microorganisms, natural yeasts have been widely used as biological control agents (Irtwange, 2006; Qing and Shiping, 2000). Yeasts possess many properties that make them useful for control purposes. Yeasts generally do not produce allergenic spores or mycotoxins as many mycelial fungi do or as antibiotic metabolites likely to be produced by bacterial antagonists (Droby and Chalutz, 1994). Yeasts generally have simple nutritional requirements and are able to colonize dry surfaces for long periods of time, as well as withstand many pesticides used in the postharvest environment (El-Tarabily and Sivasithamparam, 2006; Wilson and Wisniewski, 1989). In addition, yeasts can grow rapidly on inexpensive substrates in fermenters and are therefore easy to produce in large quantities (Druvefors, 2004). Past studies and suggested modes of action of biological control yeasts indicate less likelihood of any hazard to consumers (Arras and Arru, 1997; Arras *et al.*, 1999). Furthermore, yeast cells contain high amounts of vitamins, minerals and essential

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amino acids and there are several reports on the beneficial effects of yeast in foods and feeds (Hussein *et al.* , 1996).

The modes of action of yeast antagonists may give rise to the answer as to whether the yeast could be used as biological control agents. Several possible modes of action of the antagonistic yeasts have been suggested, i.e., competition for space and nutrients, production of cell wall-degrading enzymes (β-1, 3-glucanase and chitinase), killer toxins production, stimulation of host defense responses (Janisiewicz and Korsten, 2002). However, there is evidence that the mode of action of several yeast species used as biological control agents does not involve antibiosis but rather competition for nutrients at the wound site (Chand-Goyal and Spotts, 1996; Droby, and Chalutz, 1994). For *Pichia anomala*, the production of cell wall lytic enzymes and killer toxins has been suggested to be responsible for antifungal activity against *Botrytis cinerea* (Jijakli and Lepoivre. 1998). Studies on the distribution of radiolabelled glucose between the antagonistic yeast *Sporobolomyces roseus* or *Cryptococcus laurentii* and the pathogen *B. cinerea* show a strong sugar assumption by the yeast that blocks fungus conidial germination due to deprivation of nutrients (Filonow, 1998). In fruit wounds, competition for nutrients is probably extended to other nutrient, such as nitrogen compounds present in low concentration. Moreover, *Pichia guilliermondii* and *Debaryomyces hansenii* have been shown to attach directly to the cell walls of postharvest pathogens *Penicillum italicum* for nutrient competition (Arras *et al.* , 1998; Droby *et al.* , 1989). They also involved the lysis of *B. cinerea* by β-1, 3-glucanase (Wisniewski *et al.*, 1991). Other possible modes of action of the yeast antagonists have also been suggested. *Cryptococcus laurentii* has been shown to be resistance to oxidative stress caused by the reactive oxygen species in apple wounds infected by *Botrytis cinerea* and *Penicillium expasum*, and thus they could out compete the wound pathogens (Castoria *et al.*, 2003). *Pichia membranefaciens* has been shown to induce several proteins related to host defense mechanisms, e.g. antioxidant proteins, pathogenesis related proteins, and enzymes associated with sugar metabolism in peach fruit infected by *P. expasum* (Chan *et al.*, 2007). In addition, the expression of genes related to plant host defense, i.e., the cell wall degrading enzyme produced by β-1,3-gucanase genes has been suggested to involve in the mode of action of *Cryptococcus laurentii* in controlling postharvest disease of jujube fruit (Tian *et*

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al., 2007). However, the preliminary studies on modes of action of the yeast antagonists for biological control in the new situations e.g. types of pathogen or plant host as in our case are still essential for supporting the further deeper investigation, i.e., molecular or proteomic levels. Therefore, identification of modes of action of yeast biological control agent is very important both for regulatory approval and for optimizing the biological control system.

Anthracnose caused by *Colletotrichum capsici* is one of the major diseases of tropical vegetables such as chilli (*C. annuum* L. var. *acuminatum* Fingerh.) (Hadden and Black, 1989; Pakdeevaraporn *et al.*, 2005). This disease appears as ripe fruit-rot and die-back (Mehrotra and Aggarwal, 2003). Ripe fruit-rot is more conspicuous as it causes severe damages to mature fruits in the field as well as during transit and storage. The first outward sign of infection is the appearance of a small, black, circular spot, generally sharply defined or at times diffused. Die back usually appears after rain or after a prolonged dew period. The attack commences from the growing point of the flower bud, the tops of the affected branches wither and turn brown. The infected plants bear fewer fruits of low quality. Under conditions favorable to disease development, up to 50% of the fruits can be damaged (Smith and Crasson, 1959). Typically, symptoms first appear on mature fruits as small, water-soaked, sunken lesions that rapidly expand. The lesion may increase to 2-3 cm in diameter on large fruits. *C. capsici* produces conidia in an acervulus and the spore dimension is 17-18 × 3-4 μm. The appressoria from germinated conidia then penetrate into chilli epidermal cells. Mycelial growth inside host tissues produces a thermostable toxin which causes injury, particularly to the protoplasmic content of the host tissues (Narain and Das, 1970).

Demand for chillies in the world is increasing every year (Food and Agricultural Organization of the United Nations, 2004) and good quality chillies are required, i.e., absence of diseased appearance or fungal toxins are prerequisites for import and export (The Chile Pepper Institute, 2004). However, control of anthracnose disease on chilli fruits still relies mainly on the use of synthetic fungicides (Madden *et al.*, 1993). This may result in fungicide-resistant pathogens as occurred in copper fungicides and dithiocarbamates (Parry, 1990). In addition, there is also a problem of fungicide contamination in foods and the environment. Thus there is an increasing

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need for alternative control strategies. Several research works have shown successful use of yeast antagonists to control plant pathogens in fruits (Wilson *et al.*, 1993; Lurie *et al.*, 1995).

This scientific research aims to investigate the possibility of using harmless yeast biological control agent(s) of chilli anthracnose in order to reduce the use of chemical agents that may be harmful to humans and the environment. In this study, epiphytic yeast strains from fruits and vegetables have been isolated and identified. Their capabilities to control anthracnose disease caused by *C. capsici* were investigated. Moreover, interactions between yeast cell, fungal hypha and plant tissue were studied in order to elucidate the modes of action of the effective yeast antagonist, i.e., competition for nutrients, secretions of fungal cell wall-degrading enzymes, and killer toxin production.

Educational approach

In the past decade, an increasing number of both national and international reports indicate a growing concern over the effectiveness of education teaching practices and the decreased science (and math) performance of students relative to occupational demand (Project Kaleidoscope, 2006; Asian Development Bank, 2003). A variety of national stakeholders, including business and educational leaders, politicians, parents, and public agencies, have called for long-term transformation of the educational system to produce graduates who are well trained in science, can engage intelligently in global issues that require local action, and in general are better able to solve problems and think critically. Specifically, business leaders are calling for graduates who possess advanced analysis and communication skills, for instructional methods that improve lifelong learning, and ultimately for an educational system that builds a nation of innovative and effective thinkers (Business-Higher Education Forum and American Council on Education, 2003). Education leaders are similarly calling for institutions to produce graduates who can think critically, communicate effectively, and who employ lifelong learning skills to address important scientific and civic issues (Association of American Colleges and Universities, [AACU] 2005; Office of National Educational Commission [ONEC], 1999).

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Many educational institutions consider critical thinking to be one of the most important indicators of student learning quality (AACU, 2005; Department of Curriculum and Instruction Development, 2002; Ennis, 1987). Teaching to support the development of critical thinking skills has become a cornerstone of nearly every major educational objective since Ministry of Education in many countries has made efforts to bring about genuine and effective of educational reform. Educational organizations have tried to make a fundamental shift from traditional teacher-focused instruction to more student-centered learning that encourages discovery, reflection, and in general is thought to improve student critical thinking skills (Quitadamo and Kurtz, 2007). In Thailand, the Institute for the Promotion of Teaching Science and Technology (IPST) has supported this trend with recommendations to improve the advanced thinking skills that support scientific literacy (IPST, 2001). Critical thinking is very close to scientific thinking because when one uses the scientific method to study or investigate nature or the universe, one is practicing scientific thinking. If one uses the methods and principles of scientific thinking in everyday life such as seeking solutions to problems of economics or philosophy, or just trying to answer personal questions about oneself or the meaning of existence, one is said to be practicing critical thinking. This skill means thinking correctly for oneself that successfully leads to the most reliable answers to questions and solutions to problems (Schafersman, 1997). More recent reports describe the need for improved biological literacy as well as international competitiveness (Klymkowsky, 2006). Therefore, it is necessary to promote the research-supported teaching and learning practices, which can help students to develop the cognitive science that is a goal of science learning (Bybee and Fuchs, 2006; Malcom *et al.*, 2005).

Moreover, ONEC (1999) has guided the learning process organized activities for learners to draw from authentic experience; drill in practical work for complete mastery and enable learners to think critically. Many research works have shown that people construct knowledge from their experiences (Baker, 2002; Kayes, 2002). The process of experiential learning is especially important in childhood learning, where children need sensual and physical activity to draw knowledge from it. Experiential education is not a new movement in the field of education. There are numerous scholars who have proposed learning models with the characteristics of experiential

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learning (Wulff-Risner and Stewart, 1997). John Dewey is considered to be one of the most influential educational theorists of the twentieth century. As early as 1938, Dewey believed that "there is an intimate and necessary relation between the processes of actual experience and education" (Dewey, 1938). Dewey believed that textbook problems most often were not real problems to students and that school learning should be an experientially active, not passive affair (Kolb, 1984). Dewey supported learning experiences in which learners are directly in touch with the realities being studied, rather than simply reading about, hearing about, or talking about these realities. When experiential learning techniques are used as contributors to the creation of a learning environment that maximizes learners' skills in learning from their own experiences, the full potential for learning can be realized (Kolb and Fly, 1975). Experiential learning activities can be developed in many situations. One situation in agricultural programs involves the teaching of pest control evaluation or judging skills (Wulff-Risner and Stewart, 1997). Judging implements the experiential learning process and concept are "do, apply, and reflect". The student experiences the judging situation, reflects on the information available, processes that information, generalizes concepts previously learned to the situation, and finally makes a decision by applying concepts learned. However, there are insufficient experiential learning environments which involve the senses and physical activity (Kritzenberger *et al.*, 2002). Therefore, this study aims at applying the results from the research works to developing experiential learning for secondary school students.

The experiential learning on microbial control of plant disease is one of the strategies for conceptual understanding of organism interrelationships, especially, plant and microbe. These relationships has been defined in many ways; parasitism, competition, saprophytism, mutualism, etc. One example of the microbial control of plant diseases is yeast biological control, a technique used widely in agricultural sector nowadays (Clive, 2006). The basis of this technique was developed from the knowledge on interactions among plant host, fungal pathogen and epiphytic yeast. Moreover, yeast biological control is an alternative way to replace method using synthetic chemical, which may be harmful to living organisms and the environment. Therefore, the integration of hands-on yeast biological control experiment into the biology course promises to be an effective strategy for enhancing conceptual

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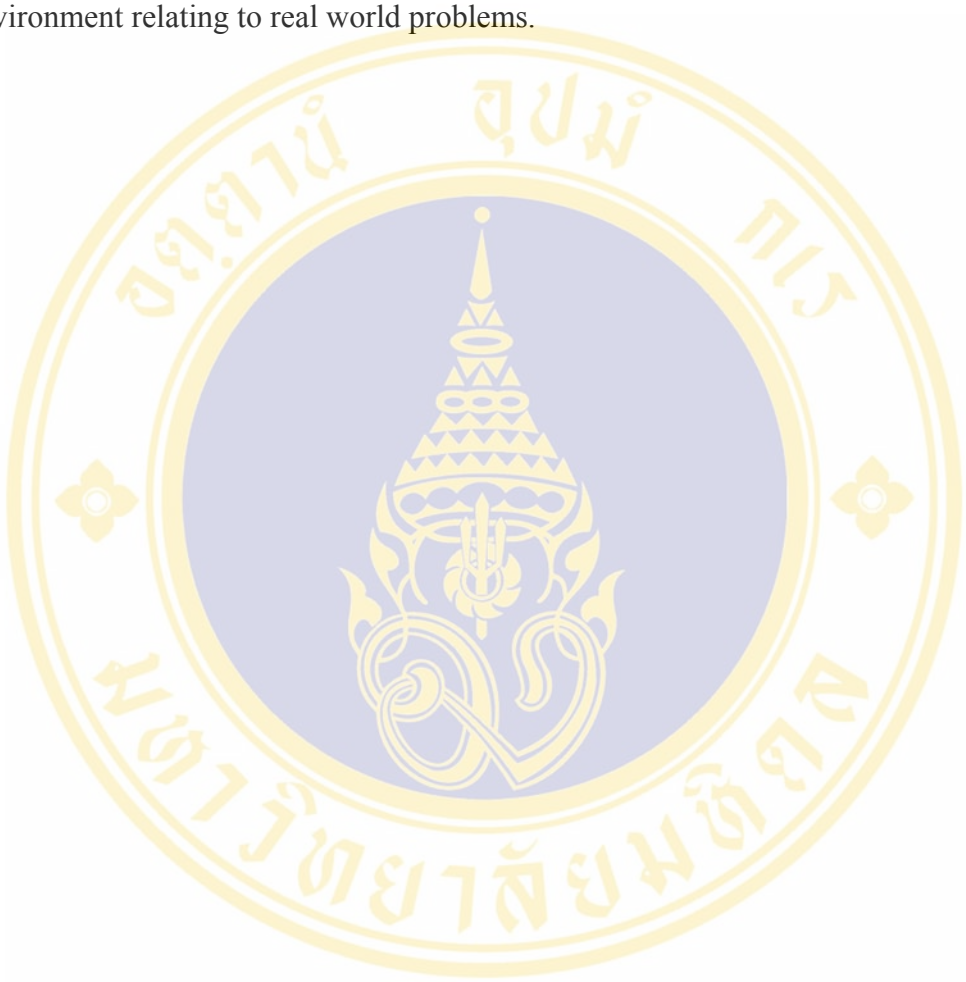
understanding of organism interrelationship and critical thinking skills on the impact of chemical fungicides used in agriculture system. Based on this rationale, the experiential learning on yeast biological control has been implemented in Thai high school students to enhance cognitive learning and provide effective learning environment relating to real world problems.



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CHAPTER II
OBJECTIVES

This chapter involves both scientific and educational objectives. The possibility of using yeast biological control for postharvest chilli anthracnose caused by *Colletotrichum capsici* has been investigated. The results of scientific investigation were transformed into experimental learning model for secondary school students to enhance conceptual understanding of biological control and promote critical thinking skills.

Scientific purposes

The environmental impact problems from chemical fungicides are now increasing worldwide. Regulatory agencies have reacted to public pressure and introduced comprehensive legislation to reduce fungicide use (Mitchell, 1992). The limited number of viable alternatives to synthetic fungicides, however, is currently the major obstacle in reaching that goal. There is also the absence of effective chemicals for the control of many diseases. Some fungal diseases have developed resistance to certain fungicides (Irtwange, 2006). Biological control of postharvest diseases has been one of the most extensively studied alternatives and appears to be a viable technology. Research and development of biological control products for postharvest use has been on a fast track (Pal and Gardener, 2006). Yeast antagonist is one of the effective biological control agent isolated from the surface of plants and appeared to be very well adapted to growth and colonizing fruit and vegetable surfaces (Smilanick, 1994). This feature suggests that these yeasts provide an excellent means of delivering biological control agents. Possible modes of action of yeast biological control agents have been suggested e.g. secretions of fungal cell-wall-degrading enzymes, killer toxin production, competition for nutrients and areas where pathogen germinate and infect the plant tissue (Irtwange, 2006; Druvefors, 2004). Some effective yeast antagonists have already been identified and patented (Fravel, 2005).

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Among postharvest disease that damaged the crops, anthracnose is one of the most important tropical diseases of many crops included chilli (*Capsicum* spp.) (Pakdeevaraporn, 2005). This disease caused by several *Colletotrichum* spp. *Colletotrichum capsici* and *C. gloeosporioides* are two significant causal pathogens in Thailand as well as in Korea (Kim *et al.*, 1989), and United States, i.e., Louisiana (Hadden and Black, 1989) and Mississippi (Roy *et al.*, 1997). Chilli fruit is one of the important ingredients in food worldwide, thus it is important to keep it free from pest and disease damages in order to preserve their qualities, i.e., colour, flavor, spice, and nutritional value. From these rationales, the scientific research aimed to control postharvest chilli anthracnose by using natural occurring yeast antagonists.

The objectives of this part are as follows:

1. To screen for yeast strains from fruits and vegetables that can control postharvest anthracnose disease of chilli fruits caused by *C. capsici*, as judged by their abilities to inhibit the fungal growth both *in vitro* and *in vitro*.

2. To identify the effective antagonistic yeast strains capable to control postharvest chilli anthracnose by using rDNA sequence technique corraborated with their morphological and physiological characteristics.

3. To study the effects of concentrations of the effective antagonistic yeast strains, storage temperatures, and storage times on postharvest decays of chilli fruits. Results from this step will be used as valuable and reliable information for an effective use of the selected antagonistic yeast as biological control agent for anthracnose disease in chilli fruits.

4. To study the interaction between the effective yeast antagonist and the fungal pathogen, *C. capsici*, on the wound of chilli fruit.

5. To study the modes of action, i.e., nutrient competition, secretion of hydrolytic enzymes, killer toxin production. The results will be used to support their uses as biological control agents.

Educational purposes

In science education, hands-on activities in classrooms possess a high potential in facilitating the learning process. One of the advantages of the hands-on in education is that they can draw students and teachers into the real world of science. In

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many schools, there is a tendency for students to sit in classes and to learn from books, where things are written down and underlined properly and where the answer in the back of the book. However, in a real laboratory, the answer isn't in the back of the book (Cole, 1985). Unfortunately, there is still a lack of effective designed experiments that can both relate to the real world and well develop cognitive learning (Fenwick, 2001). Recently, international studies, i.e., the Third International Mathematics and Science Study (1996) and the Programme for International Student Assessment (2006), have pointed to a specific need to improve science teaching, scientific skills, and critical thinking skills of students. Deficiencies in effective experimental activity may in particular explain students' failures to understand scientific concepts (Baumert *et al.*, 1997). Those deficiencies may arise from a low level of methodological competencies (Mayer, 2002). A balanced ratio of theory to practice in teaching approaches seems to lead to appropriate scientific understanding, but practice in science teaching currently seem to favor the theory and tend to neglect practical experience (Euler, 2001). Consequently, teaching science should involve more laboratory work. However, the reality of the school situation provides scant opportunity for hands-on experimentation, especially in biology (Ottander and Grelsson, 2006). Experimentation has been proved to enhance students' understanding of scientific concepts or students' problem solving abilities (Hofstein and Lunetta, 2004).

In addition, Quitadamo and Kurtz (2007) have suggested that if instructors wanted students to think critically about science, they would need to use teaching methods that improved students' critical thinking performance. In many traditional biology courses, student were not expected to work collaboratively, to think about concepts as much as memorize facts, or to develop and support a written project or argument. This has presented a large problem when one considers the societal role that students will play as voter, community members, and global citizens. By improving their critical thinking skills in science, students will be better able to deal with the broad scientific, economic, social, and political issues they will face in the future.

Joplin (1981) has suggested an experiential education activity as one where a real life, work-based experience was followed by an opportunity for a structured reflection on the experience. The structured reflection helps a learner understand the

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implications of the activity and how it can be applied to more general situation. More recent literatures equipped with testimonials, journals, case studies, and other data have indicated the positive impact of experiential course in school and university, i.e., developing the life skills of critical thinking and decision making (Kraft and Kielsmeier, 1995; Wulff-Risner and Stewart, 1997).

In general, the curriculum of biology in high school desires students to develop an understanding in the concept of organism interrelationships (National Research Council, 1996; IPST, 2001). However, there is still a lack effective hands-on activity that can provide direct experience for students to study such as relationship in the natural habitat. Traditional teaching by lecture or paper activity in the class often resulted in failure of conceptual understanding of students. Moreover, student cannot relate the matter to everyday life and do not realize the import on their society. Knowledge on biological control of plant pests and pathogens can inspire research and development in many other fields (McSpadden and Fravel, 2002). Biological control means suppressing pest organisms or pathogens with other organisms, however, the interrelationships of many environmental variables can result in multiple interactions among organisms and their environment. The biological control concept can be well connected between school biology knowledge and real world phenomena.

The educational part of this research aims at adapting the findings from the scientific part on yeast biological control of anthracnose disease to construct an experiential learning model to enhance students' conceptual understanding on biological control and promote critical thinking skills of students.

The objectives of this research are as follows:

1) To develop an attractive and easy hands-on experiment of yeast biological control for secondary school student

2) To implement this experimental learning model to the students in comparison with the traditional method

3) To construct instruments for assessment of students’ conceptual understanding, critical thinking skills, and student attitudes toward yeast biological control experiment

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4) To compare learning achievements (conceptual understanding and critical thinking skills) of students taught by experiential learning model to that by traditional method

5) To evaluate students' attitudes toward the hands-on yeast biology experiment

6) To promote student's awareness of environmental preservation with inspired biological control article and questioning

Research questions

Both scientific and educational parts of this study aims to answer the following questions:

1. Will yeast isolates from surface of fruits and vegetables be able to control anthracnose disease in chilli fruits caused by *C. capsici*?
2. What are the species of the effective antagonistic yeasts?
3. How does the most effective antagonistic yeast act to control anthracnose disease in *C. capsici* infected chilli fruits?
4. Will the experiential learning model on yeast biological control enhance student's conceptual understanding?
5. Will the experiential learning model on yeast biological control enhance students' critical thinking skills?
6. What are the attitudes of students towards hands-on activities on yeast biological control?

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CHAPTER III
LITERATURE REVIEW

This chapter presents literature reviews on both scientific and educational aspects. The scientific part covers the importance of biological control, biological control agents, plant pathogen, and the role of yeast in biological control. Anthracnose disease in chilli as caused by *Colletotrichum capsici* has also been mentioned. The educational part reviews certain problems in learning and teaching biology, and examples of the model used in education, with special emphasis on experiential learning model. Tools used in assessment of learning achievements in this study have also been reviewed.

Scientific review

Postharvest diseases cause losses yearly in the fruit and vegetable productions worldwide. Fungicides, the major weapon in combatting these diseases, are often ineffective and pose hazards to humans and the environment. Therefore, there is a critical need for new methods to control postharvest diseases. Yeast control of postharvest diseases has been studied extensively as one of the alternatives and appears to be a viable technology. Research and development on yeast biological products for postharvest use has been on a fast track. Several commercial products are already available and others will be available in the near future. The opportunities for successful implementation of this technology are growing as information regarding various aspects related to formulation technology, application, microbial ecology and genetics increases.

3.1 Biological control of postharvest fruits and vegetables diseases

Fruits and vegetables are considered important to human health because they supply essential nutrients including vitamins, minerals, and other necessary compounds such as antioxidants (Janisiewicz and Korsten, 2002). Increased consumer awareness in linkage between diet and health has therefore resulted in a greater consumption of fruits and vegetables. At the same time, however, consumers are

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equally concerned about safety of the fruits, i.e., free from pesticide residues, toxins and harmful microorganisms. Losses due to pests and diseases in the field, during storage, as well as in transit and commercialization can amount up to 25% of the total production in industrialized countries (Spadaro and Gullino, 2004). In developing countries the damages are often higher, exceeding 50%, due to inadequate postharvest control (Karabulut and Baykal, 2003).

To control the postharvest diseases, it is helpful to understand nature of the disease organisms, conditions promoting their occurrence, and factors affecting their actions. Bacteria and fungi are the causative agents of postharvest diseases, the latter is more common in both fruits and vegetables (Tournas, 2005). Postharvest diseases caused by bacteria are rare in fruits and berries but somewhat more common in vegetables (Boyette *et al.*, 2000). Viruses seldom cause postharvest diseases, although they, like postharvest disorders, may weaken the produce. Most postharvest fungal diseases (rots) are caused by the dispersion of tiny spores formed by the actively growing pathogen. Spores have adapted to survive in hot, cold, or very dry conditions, and may be carried a great distances by wind or water and can cover most exposed surfaces in great numbers. Spores may remain dormant for a long period until they meet the suitable conditions for germination and growth. These conditions include water (in liquid form or as high relative humidity), warm temperature, low light level, adequate levels of oxygen and carbon dioxide, and the presence of nutrients in the form of sugars, starches, or other organic compounds. Spores of postharvest fungal pathogens are most susceptible to chemical control while they are germinating to produce actively growing mycelium. Under the appropriate circumstances, germination often takes place in only a few hours. Once the active growth is under way and the organism moves below the surface of fruit or vegetable, chemical control becomes very difficult (Boyette *et al.*, 2007).

Many immature fruits and vegetables contain compounds that inhibit the growth of some disease organisms (Bachmann and Earles, 2000). These compounds and the resistance they provide are often lost during ripening. Therefore, a fresh wound on the surface of a warm, wet, ripened fruit or vegetable enclosed within a shipping container provides an ideal site for postharvest pathogens to colonize and develop. Gentle handling to prevent wounding and thorough cooling immediately after

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harvest can significantly reduce the incidence of postharvest disease. The potential for inoculation and infection by postharvest pathogens is always present when handling fresh produce. Understanding the several different ways by which these organisms can come into contact with the produce can be helpful in formulating the control measures.

Although, synthetic fungicides have been used as the primary means to control postharvest diseases (Wilson and Wisniewski, 1989), however, at present, several issues arisen that lead to searching for alternative approaches. These are public's growing concerns for the human health conditions and the environmental pollution associated with pesticide usage in orchards (Wilson and Wisniewski, 1994), the development fungicide-resistant strains of postharvest pathogens (Romano *et al.*, 1983; Spotts and Cervantes, 1986) and the lack of continued approval of some of the most effective fungicides (Gullino and Kuijpers, 1994). Biological control fits in well with the concept of sustainable agriculture because it mostly exploits natural enemies of pests and plant pathogens with reduced environmental impact. Among the biological strategies applicable to postharvest, the induction of resistance in fruits and the use of plant or animal products with a fungicidal activity, resulted in the application of antagonistic microorganisms have been seriously considered (Janisiewicz and Korsten, 2002). Biological control using antagonists has proved to be one of the most promising alternatives, either alone or as part of an integrated pest management policy to reduce pesticide use (Wilson and Wisniewski, 1994).

During the past 20 years, several biological control agents have been exploited and widely investigated against different postharvest fungal pathogens, like *Aspergillus* spp., *Botrytis,* spp., *Monilia* spp., *Penicillium* spp., and *Rhizopus* spp. (Droby *et al.*, 2001; Lima *et al.*, 1997; Northover and Zhou, 2002; Viñas *et al.*, 1998; Zahavi *et al.*, 2000). Many of the early studies aimed at mode of action and efficacy of some potential biological control bacteria, such as *Brevibacillus subtilis*, producers of antibiotics (Pusey *et al.*, 1986), however, the application of such bacteria on fruits did not prove to be commercially acceptable. Characteristics of an ideal antagonist as suggested by Wilson and Wisniewski (1989) are as follows: genetic stability, efficacy at low concentrations and against a wide range of pathogens on various fruit products, simple nutritional requirements, survival in adverse environmental conditions, growth on cheap substrates in fermenters, lack of pathogenicity for the host plant and no

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production of metabolites potentially toxic to humans, resistance to the most frequently used pesticides and compatibility with other chemical and physical treatments. Yeast seems to possess a good number of the above-mentioned features (Druvefors, 2004). During the last few years, many research works have focused on the selection and characterization of the potential yeasts (Chalutz and Droby, 1998).

3.2 Antagonistic microorganisms

A number of microorganisms (bacteria, yeasts and fungi), which effectively control postharvest pathogens, have been identified for postharvest control (Wilson and Wisniewski, 1989; Wisniewski and Wilson, 1992); some of these have been patented and registered (Mendelson *et al.*, 1994). Antagonists are often isolated from the surface of plants (Smilanick, 1994). For example, searching for antagonists on healthy apples in the orchard and storage, "a place where a disease can be expected but it does not occur" (Baker and Cook, 1974), resulted in isolation of many ecologically fit bacterial and yeast antagonists effective against postharvest decays of apple (Janisiewicz, 1987; Janisiewicz, 1991). Similar isolations from other fruits also yielded effective antagonists against postharvest fruit decay pathogens (Adikaram and Karunaratne, 1998; Zahavi *et al.*, 2000). Isolation of the antagonists can be improved by using fruits from unmanaged orchard (Falconi and Mendgen, 1994; Janisiewicz, 1996), where natural populations have not been disturbed by chemical usage, and the pool of potential antagonists is greater than in a chemically managed orchard (Smolka, 1992). A variety of enrichment procedures have been used that favor isolation of microorganisms growing efficiently on the substrate, which occurs at the infection site (wound) that must be protected. These include isolation from natural cracks on the fruit surface (Janisiewicz and Korsten, 2002); agar plates containing apple juice seeded with fruit washings (Janisiewicz, 1991); fruit wounds treated with fruit washings and incubated for several days (Wilson *et al.*, 1993); freshly made wounds on apples in the orchard that were exposed to colonization by fruit-associated microorganisms for one to four weeks before harvest (Janisiewicz, 1996); and from an apple juice culture seeded with the colonized wounds inoculated apple juice (Fillow, 1998).

Fruit surface has provided the most abundant and most desirable source for isolating antagonists against postharvest fruit pathogens (Pal and Gardener, 2006).

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However, the antagonists may also come from other closely related or unrelated sources. Leaf surface has also been a good source of antagonists, as it may share part of the resident microflora of fruits as well as contain other microorganisms dislodged from the fruit (Falconi and Mendgen, 1994; Korsten *et al.*, 1997). Starter cultures used in the food industry (Pusey, 1991) may also yield effective antagonists. Soil may also be an abundant and diverse source of antagonists. *Bacillus subtilis* B3, a contaminant found during routine isolation of fungi from peach roots and identified for its inhibition of *M. fructicola* on agar plates (Janisiewicz and Korsten, 2002), is a very effective strain for biological control of brown rot of peach (Pusey and Wilson, 1984). B3 was the first strain studied extensively for biological control of postharvest diseases. The early successes in biological control of brown rot on peach with this strain (Pusey and Wilson, 1984) has stimulated interest in biological control of postharvest diseases of fruits in general.

3.3 Yeast biological control

There are many yeasts and yeast-like organisms for control of postharvest citrus rot (Wilson and Chalutz, 1989; Droby *et al.*, 1989; Droby *et al.*, 1991; Wisniewski *et al.*, 1991). Both naturally occurring and artificially introduced antagonists have been proposed as promising alternatives to fungicide control of post-harvest diseases (Wilson and Wisniewski, 1989; Wisniewski and Wilson, 1992). Some examples of yeast isolates used as biological control agent include *Cryptococcus infirmominiatus*, a biological control of naturally-occurring yeast, to control decay of sweet cherry either alone or in combination with a fungicide and modified atmosphere packaging (Wilson and Wisniewski, 1994). Several postharvest diseases affect sweet cherry, including blue mold (*Penecillium expansum*) and brown rot (*Monilinia fructicola*). *C. infirmominiatus* alone did not control brown rot in air-stored cherries, but it did control blue mold, probably because the longer germination time of blue mold conidia allowed the yeast enough time to grow and colonize wound sites to prevent infection. A single preharvest application of the fungicide iprodione reduced brown rot, but better control was achieved when cherry fruit were also treated with a postharvest dip in the beneficial yeast suspension (Janisiewicz and Korsten, 2002). The combination of preharvest fungicide and postharvest biological control reduced decay by 88-97%. Modified atmosphere packaging also reduced brown rot, with the

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greatest reduction when used in conjunction with treatment with the beneficial yeast. Combining all three treatments in an integrated control strategy reduced brown rot from 41% to less than 1%. This strategy gives excellent decay control during long-term storage and transport of sweet cherry fruit (Aharoni, 2004).

Another yeast isolate from peach fruit is *Pichia membranefaciens*, which at concentration of $5x10^{8}$ cells per ml completely inhibit Rizopus rot in nectarine wounds artificially inoculated with $5x10^{4}$ spores per ml upon storage at 15, 25, and 30 ^{o}C. The use of *P. membranefaciens* for biological control is compatible with several common postharvest practices including fungicide, calcium treatment, and cold storage (Tian *et al.*, 2002). Other examples of antagonistic yeasts used for bicontrol agents of various fruits and vegetables are shown in the Table 3-1.

Table 3-1 Yeast species containing biological control activity.

Biological control species	System	Target organism	References
Cryptococcus albidus	bean, tomato, strawberry, *in vitro*	*Bortytis cinerea* *Penicillim glabrum*	Elad, *et al*,1994; Helbig, 2002;
Cryptococcus laurentii	apples	*Penicillium expansum* *B. cinerea*	Lima *et al.* , 1998; Vero *et al.* , 2002
Debaromyces hansenii	oranges, apples, grapefruit	*P. digitatum*	Arras, 1996; Droby *et al.* , 1989
Filobasidium floriforme	apples	*B. cinerea*	Filonow *et al.* , 1996
Kluyveromyces marxianus	*in vitro*	*P. glabrum*	Sinigaglia *et al.* ,1998
Metschnikowia pulcherrina	*in vitro*, apple	*P. glabrum* *P. expansum* *B. cinerea*	Janisiewicz *et al.* , 2001; Piano *et al.* , 1997
Pichia burtonii	seed	*Penicillium verrucosum*	Ramakrishna *et al.* 1996
Pichia guilliermondii	tomato, soy bean, grapefruit, apple	*B. cinerea* *Aspergillus flavus* *P. digitatum*	Saligkarias *et al.* , 2002; Paster *et al.* , 1993; Droby *et al.* , 1990
Pichia membranaefaciens	grapevine	*B. cinerea*	Masih and Paul, 2002
Rhodotorula glutinis	bean and tomato plants, apples	*B. cinerea* *P.expasum*	Elad *et al.* , 1994; Lima *et al.* , 1998
Saccharomyces cerevisiae	*Pinus sylvestris*	*Wood decaying fungi,* *Alternaria alternata*	Payne *et al.* , 2000 Suzzi *et al.* , 1995
Sporidiobolus salmonicolour	apples	*B. cinerea*	Filonow *et al.* , 1996
Yarrowwia lipolytica	apples	*B. cinerea*	Filonow *et al.* , 1996

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3.4 Yeast as the commercial biological control products

Yeast biocontrol agents are currently available on the market for combating postharvest decays in fruits (Table 3-2). Aspire (Ecogen Inc., Langhorne, Pakistan, USA) is based on the yeast *Candida oleophila* and has been used as a spray or dip against postharvest diseases on pome and citrus fruits (Janisiewicz and Korsten, 2002; Janisiewicz *et al.*, 2003). The product was introduced commercially in the United States in 1996. The commercial product Yield Plus with *Cryptococcus albidus* as the active antagonist was introduced commercially on the South African market in 1997 by Anchor Yeast (Anchor Yeast (Pty) Ltd, Cape Town, South Africa). Yield Plus has been used as a biological control product against *Botrytis*, *Penicillium* and *Mucor* on, strawberry, apple and grape and is also under evaluation for other crops (Lisa Picard, Anchor Yeast, Cape Town, South Africa, pers. comm.). The recent product Shemer, registered in Israel, based on a newly identified yeast *Metschnikowia fructicola* (Kurtzman and Droby, 2001), is effective against a wide range of pathogens of grape, strawberry and sweet potato (M. Keren Zur, Agrogreen Minrab Group, Ashdod, Israel, pers. comm.)

Table 3-2 Commercial yeasts as biological control agents

Biological control agent	Target organisms	Crops	Product	Manufacturer or distributor
Candida oleophila	*Botrytis, Penicillium, and Geotrichum*	citrus, grape fruit, and apple	Aspire	Ecogen, Pakistan, USA
Cryptococcus albidus	*Botrytis*, *Penicillium* and *Mucor*	apple, strawberry, and peach	Yield Plus	Anchor Yeast, South Africa
Metschnikowia fructicola	*Botrytis*, *Rhizopus, and Sclerotinia*	grape, strawberry, and sweet potato	Shemer	AgroGreen, Israel

The commercial development of Aspire by Ecogen-Israel Partnership, Ltd. firstly focused on the biological control of postharvest decays of citrus, mainly by blue mold and green mold caused by *P. itallicum* and *P. digitatum*, respectively, which

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invade through wounds after harvest. The early research and the pilot test in commercial packinghouses have been conducted with a yeast biological control agent, *P. guilliermondii* (originally described as *Debaromyces hansenii*) (MacLaughlin *et al.*, 1990). Results indicated that a combination of the yeast with a tenfold diluted commercial rate of thiabendazole (TBZ) provided pathogen control equal to the full-strength fungicide. Subsequently, the focus has been changed to the yeast antagonist *Candida oleophila*, previously identified as *C. sake* (Wilson *et al.*, 1993). Tests in commercial citrus packinghouses on oranges and grapefruit in Israel indicated that, like *P. guilliermondii*, *C. oleophila* gave satisfactory control of green and blue mold and sour rot caused by *Geotrichum candidum* only in combination with tenfold diluted TBZ (Droby *et al.*, 1998). Perhaps a more effective antagonist could have been found if more emphasis was put on preliminary and secondary screening at the initial stage of research, and on selection criteria for antagonist designated for commercial development. In addition, changing the biological control agent during commercialization may have had an effect on the final product, as less extensive studies with the new antagonist were conducted. It is necessary to take into account as many aspects of biological control prior to commercialization as possible; hasty commitment to biological control agents without appropriate testing and classification should be avoided. The citrus system seems less prone to biological control than the pome fruit system. Combining yeasts with other control methods (Wilson and Ghaouth, 1993) appears to be a sensible approach at this time, although the citrus system may benefit greatly from more rigorous selection and manipulation of the antagonists to obtain a strain with enhanced biological control activity.

Yield Plus or yeast *Cryptococcus albidus*, originally isolated from mature strawberry fruits, was tested for antagonistic activity against *Botrytis cinerea*, the causal agent of grey mould in strawberries (Wisniewski *et al.*, 1991). Conidial germination and germ tube growth of conidia of *B. cinerea* were inhibited by a cell suspension of the antagonist in aqueous strawberry fruit pulp suspension (1%) after 6 and 24 hours of incubation. Application of a cell suspension (1×10^6 cells ml^{-1}) on detached strawberry leaf disks incubated at 10°C reduced incidence and conidiophore density of *B. cinerea* by 86 and 99%, respectively, however, the effectiveness was reduced at higher temperatures. Treatments of strawberries in bloom with *C. albidus*

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reduced incidence of grey mould on ripe strawberry fruits after harvest by 33, 28 and 21% in three years of field trials (Helbig, 2002). The effectiveness of the yeast was increased when formulation substances (alginate, xanthan and cellulose) were added to the cell suspension (Elmer and Reglinski, 2006).

Another commercial yeast biological control product, Shemer is based on the yeast *Metschnikowia fructicola*. Its mode of action is believed to be mainly through competition, with no involvement of antibiotic substances, and therefore has a minimal effect on the environment (Kurtzman and Droby, 2001). The commercial product (water-dispersible granules) is stable under ambient storage, and can be applied through spray or drench application systems in the field or in packinghouses. Shemer has been registered in Israel and tested under commercial field and packinghouse conditions in several crops including strawberries, sweet potatoes, grapes and citrus. Over the past 2 years the effectiveness of the product has been evaluated under commercial field and packinghouse conditions against several new pathogens of fruits and vegetables. Shemer treatments in commercial packinghouses significantly reduced development of *Rhizopus stolonifer* on peaches, *Botrytis cinere*a on chilli and *Sclerotinia sclerotium* on carrots in pilot-scale tests. Application of Shemer in the field also proved useful in protecting fruits after harvest, when postharvest treatments are not practiced. Weekly applications of Shemer on strawberry reduced rot development in the field and also the incidence of gray mold (*B. cinerea*) and Rhizopus fruit rot (*R. stolonifer*) during storage (Blachinsky *et al.*, 2007). In northern Italy, application of Shemer 24 h before harvest of soft berries, reduced significantly the number of decayed berries caused by *B. cinerea*. In Chile, Shemer was found effective in reducing Botrytis rot in table grapes (Blachinsky *et al.*, 2007). In most of the trials, the level of decay control was comparable to that of the most common chemical fungicides currently used by the industry, leading to the registration of the product in Israel. Integration of Shemer with other environmentally safe approaches, including physical means (hot water wash, heat, modified atmosphere) or a variety of mild chemicals (food grade additives) increased the treatment efficacy (Agrogreen, 2006).

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3.5 Modes of action of yeast biological control agents

Information on the mechanisms of action for most of yeast antagonists investigated is still incomplete due to the difficulties encountered during the study of the complex interactions between host, pathogen, antagonist and other microorganisms present. However, several possible yeast biological control mechanisms have been suggested to be competition for nutrients and space, killer toxin effect, productions of hydrolytic enzymes or direct interaction with the pathogen, and induction of host resistance (Droby and Chalutz, 1994).

3.5.1 Competition for nutrients and space

Competition for nutrients and space has been suggested as one of the modes of action of several possible biological control yeasts, e.g. *P. guilliermondii* against *Penicillium digitatum* (Droby *et al.*, 1989), *Candida guilliermondii*, *Cryptococcus laurentii* and *Metschnikowia pulcherima* against *Botrytis cinerea* and *Penicillium expansum* (Elad et al., 1994; Piano *et al.*, 1997; Roberts, 1990; Saligkarias *et al.*, 2002; Vero *et al.*, 2002). This is in most cases a probable and reasonable explanation. However, competition for nutrients is difficult to prove, as it is usually very hard to exclude all other mechanisms. Addition of exogenous nutrients has been shown to reduce the efficacy of several biological control yeasts including *P. guilliermondii* against *P. digitatum* (Droby *et al.*, 1989). Filonow (1998) observed that the antagonistic yeasts *C. lauretii* and *Sporobolomyces roseus* had stronger sugar consumption than the pathogen *B. cinerea*. When these yeasts were mixed with *B. cinerea* conidia in sterile, diluted solutions of fructose, glucose or sucrose, or in diluted apple juice, they inhibited conidial germination compared with no-yeast controls, suggesting that competition for nutrients are only part of the inhibiting mechanisms. In a tissue culture plate system with membrane to separate the organisms, Janisiewicz (2000) has demonstrated that *Aureobasidium pullulans* consumes the amino acids and inhibits germination of *P. expansum* in apple juice.

3.5.2 Killer toxin effect

The killer yeast phenomenon was first discovered 40 years ago by Makower and Beavan (1963). They observed that certain strains of *Saccharomyces cerevisiae* produced toxins that killed sensitive strains of the same species. Initially, it was assumed that the killer yeasts only destroyed yeasts belonging to the same or

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closely related species. However, many killer toxins can affect other yeasts and even bacteria and filamentous fungi (Walker *et al.*, 1995; Izgu and Altinbay, 1997). Since its discovery, intensive investigations on the killer system, mainly in *S. cerevisiae*, has contributed to the understanding of eukaryotic cell biology, *e.g.* virus-host cell interactions and yeast virology (Schmitt and Breinig, 2002). In addition, several applications within wine and beer fermentation (Passoth and Schnürer, 2003), food preservation (Palpacelli *et al.*, 1991) and antifungal therapy in humans and animals (Polonelli *et al.*, 1986) have been suggested. However, most killer proteins are only active within a very narrow range of temperature and pH range, which complicates application in many environments (Suzuki and Nikkuni, 1989).

3.5.3 Production of hydrolytic enzymes

Antagonist and pathogen can also interact through a direct parasitism. Wisniewski *et al.* (1991) have observed a strong adhesion of *P. guilliermondii* antagonist cells to *B. cinerea* mycelium *in vitro*, perhaps due to a lectin link. Moreover, *P. guilliermondii* shows high exo-β-1,3-glucanase and chitinase activities when cultured on various carbon sources or on the cell walls of fungal pathogens. The ability of the yeast to produce exo-β-1,3-glucanase and chitinase is hypothesized to be associated with the firm attachment of the yeast cells to fungal hyphae and the partial degradation of fungal mycelia (Wisniewski *et al.*, 1991). Grevesse *et al.* (2003) suggested the involvement of exo-β-1,3-glucanase in the biological control activity of the yeast *P. anomala* against *Botrytis cinerea* on apples. They found that the purified exo-β-1,3-glucanase from yeast culture filtrates showed an inhibitory effect *in vitro* on the germination and germ tube growth of *B. cinerea*. Addition of *B. cinerea* cell walls to a suspension of *P. anomala* has been shown to stimulate in situ exo-β-1,3-glucanase activity. In experiments with *Aureobasidium pullulans*, a yeast-like antagonist of postharvest pathogens, exo-β-1,3-glucanase activity was detected both *in vitro* and in apple wounds (Castoria *et al.*, 2001). In addition, the exo-β-1,3-glucanase activities have been found in two different antagonistic yeasts, *Rhodotorula glutinis* (LS-11) and *C. laurentii* (LS-28) grown in the presence of hyphal cell walls of the pathogens *Penicillium expansum* and *Botrytis cinerea* as sole carbon sources (Castoria *et al.*, 1997). *Pichia membranefaciens* has shown the ability to produce significantly higher levels of chitinase *in vitro* than *Candida guilliermondii* grown in Czapeck minimal

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medium (Fan *et al.*, 2002). This correlates with the observation that *P. membranefaciens* had stronger attachment capability to hyphae of pathogens than that of *C. albidus*. Bar-Shimon *et al.* (2004) has reported that production of exo-β-1,3-glucanase and chitinase activities by *Candida oleophila* was stimulated by the presence of cell wall fragments of *Penicillium digitatum* in the growth medium. Wisniewski *et al.* (1991) has shown that the yeast *Pichia guilliermondii* produced and secreted high levels of exo-β-1,3-glucanase and chitinase activities when cultured on various carbon sources or on the cell walls of fungal pathogens. The ability of the yeast to produce exo-β-1,3-glucanase and chitinase activities has been suggested to be associated with the firm attachment of the yeast cells to fungal hyphae and the partial degradation of fungal mycelia (Wisniewski *et al.*, 1991).

The gene *CoEXG1* encoding for exo-β-1,3-glucanase has been recently isolated from *Candida oleophila* (Segal *et al.*, 2002). The role of exo-β-1,3-glucanase in the biological control activity of *C. oleophila* has been investigated by generating *C. oleophila CoEXG1*-knockouts and double-*CoEXG1* transformants (Yehuda *et al.*, 2001, 2003). It was found that *CoEXG1*-knockout transformants lost their ability to secrete exo-β-1,3-glucanase into the growing medium, while the double-*CoEXG1* transformants secreted approximately twice as much exo-β-1,3-glucanase as the untransformed *C. oleophila*. In contrast, the biological control activity of *CoEXG1*-knockout and double-*CoEXG1* transformants against *Penicillium digitatum* on kumquat fruits did not differ from that of untransformed *C. oleophila* (Yehuda *et al.*, 2003). The results suggest that, under those experimental conditions, the exo-β-1,3-glucanase secreted by *C. oleophila* seem not to be essential in the biological control activity against *P. digitatum* on kumquats and it may react in an additive or synergistic manner with other genes or be active in biological control under conditions different from those examined (Yehuda *et al.*, 2003).

Through ultrastructural and cytochemical studies, El-Ghaouth *et al.* (1998) has demonstrated that *C. saitoana* yeast cells, when cultivated together with *B. cinerea* mycelium, are associated with fungal hyphae showing cytological damages, such as papillae and other protuberances in the cell wall, and degeneration of the cytoplasm. In addition, certain yeasts, such as *S. roseus* and *C. laurentii*, when applied, are able to reduce the conidial adhesion and germination of *B. cinerea* on apples,

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which is favoured by butyl acetate, a volatile aroma produced by the fruit (Filonow, 2001). These evidences support the involvement of cell wall-degrading enzymes as one of the mode of action of yeast antagonists.

3.5.5 Induction of host resistance

Several antagonistic yeasts are as effective if applied before pathogen inoculation. This observation suggests that yeast cells application induces resistance processes in the fruit skin (Pal and Gardener, 2006). Some *Candida* strains are able to cause chemical and osmotic changes in apple tissues, and thus favouring antagonist settlement (Mclaughlin *et al.*, 1990). *P. guilliermondii* strain has been shown to stimulate the production of ethylene, a hormone in grapefruit able to activate the phenylalaninammoniumlyase, an enzyme involved in the synthesis of phenols, phytoalexins and lignins (Wisniewski *et al.*, 1991). Accumulation of phytoalexins, i.e., scoparon and scopoletin has been observed in citrus fruits treated with yeast cells (Rodov *et al.*, 1994). Plant phenolic compounds are known to be antimicrobial agents, function as precursors to structural polymers such as lignin, or serve as signal molecules (Hammerschmidt, 2005). As such, this structurally diverse and ubiquitous group of plant compounds has been suggested to play a variety of roles in plant defense against pathogens as phytoalexins, structural barriers, modulators of pathogenicity, and activators of plant defense genes. These compounds act as toxins to the attacking pathogen, i.e., puncture the cell wall, delay maturation, disrupt metabolism or prevent reproduction of the pathogen (Wu and VanEtten, 2004).

3.5.6 Modes of action of yeast as biological control agent

There are different capabilities of commercial yeast biological control agents for plant disease control. Recently, the researches of these commercial products, i.e., *Candida oleophila*, *Metschnikowia fructicola*, and *Cryptococcus albidus* have been intensive investigated. The modes of action of these yeasts have been studied to support their abilities to control the diseases and to develop these biological control agents for many biological control systems.

The yeast *Candida oleophila*, the base of the commercial product Aspire, is recommended for the control of postharvest decay of citrus and pome fruit. (Paulitz and Belanger, 2001). Its modes of action include nutrient competition, hydrolytic enzyme production, and the induction of pathogen resistance in plants. The

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past researches have been shown that the yeast *C. oleophila* is capable of producing and secreting various cell wall-degrading enzymes, including exo-β-1,3-glucanase, chitinase and protease (Arras and Agabbio, 2000; Bar-Shimon *et al.*, 2004). Exo-β-1,3-glucanase and chitinase were produced and maximized in the early stages of growth, whereas protease reached a maximum level only after 6–8 days. Production of exo-β-1,3-glucanase, chitinase and protease was stimulated by the presence of cell wall fragments of *Penicillium digitatum* in the growth medium, in addition to glucose (Bar-Shimon *et al.*, 2004). The induction of pathogen resistance in grape fruit required viable yeast cells at concentrations of 10^8 to 10^9 cells ml^{-1}. Nonviable autoclaved or boiled yeast cells or lower yeast concentrations have been ineffective in enhancing fruit disease resistance (Droby *et al.*, 2001). Application of *Candida oleophila* cell suspensions to grapefruit peel tissue have been increased ethylene biosynthesis, phenylalanine ammonia lyase activity, and phytoalexin accumulation (Droby *et al.*, 2001).

The commercial yeast *Metschnikowia fructicola* has been screened and identified by Kurtzman and Droby (2001). Blachinsky *et al.* (2002) has reported that this yeast strain showed good disease control against both *B. cinerea* and *A. niger*. A water dispersible granule with a shelf life of 1 year has been successfully formulated and marketed as Shemer product in Israel by AgroGreen Minrav Group. The mode of action of this yeast has been reported to be competition for nutrients and chitinolytic activity with no involvement of antibiotic substances, and therefore has a minimal effect on the environment (Blachinsky *et al.*, 2007; Droby, 2006).

The yeast *Cryptococcus albidus*, an antagonist isolated from peach fruit, has been evaluated for biological control capability in apple against the postharvest pathogens, *Botrytis cinerea* and *Penicillium expansum* (Tian *et al.*, 2002). It was registered as commercial biological control agent (Yield Plus) recently (Wisniewski *et al.*, 1991). It has been suggested that competition for nutrients and production of hydrolytic enzymes, i.e., exo-β-1,3-glucanase and chitinase played roles in the biological control capability of *C. albidus* against these pathogens (Chan and Tian, 2005; Fan and Tian, 2001).

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3.6 Yeast identification

Yeast identification methods have been standardized to some degree since the keystone publication of Wickerham (1951). The details, however, are varied from one laboratory to another. In routine laboratory, the traditional method for identification of yeasts are morphological and physiological characteristics (Barnett, *et al.*, 2000; Kurtzman and Fell, 1998). Morphological characteristics consider yeast cell size and shape, how they reproduce vegetative (by multilateral, bipolar or unipolar budding, by fission, by forming filaments), and the form, structure and mode of formation of ascospores and teliospores, if any of these are to be found. The shape of the cells often indicates the mode of vegetative reproduction. In various genera which form filaments, certain features are used in classification, i.e., whether the septa have pores, and the occurrence and arrangement of the blastoconidia. Sexual reproductions of some yeasts are sexually by ascospores, teliospores, or basidia. Yeasts which can reproduce by teliospores are classified partly by structural features, i.e., clamp connexions, the forms taken by sporophores, and teliospores are also used. For ascosporogenous yeasts, the identification importance is given to whether asci are formed. The number of ascospores in each ascus, their shape (e.g. round, oval, hat-, kidney-, Saturn-, or needle-shaped) and whether the ascospore walls are smooth or rough, are features which are also used to separate genera, as well as to separate species within a genus. Physiological characteristics used for classifying yeasts are abilities to grow in the various nutrients and conditions, i.e., to ferment certain sugars semi-anaerobically, to grow aerobically with various compounds, each as sole major source of either carbon or nitrogen (assimilation tests), to grow without an exogenous supply of certain vitamins, to grow in the presence of 50% or 60% (usually w/v) D-glucose or 10% (w/v) NaCl + 5% (w/v) D-glucose, to grow at 37 ^{O}C, to grow in the presence of cycloheximide (actidione), to split fat, to produce starch-like polysaccharide, to hydrolyse urea, and to form acid. These characteristics are used chiefly for differentiating between species.

Combination of physiological and morphological characteristics has been used traditionally in identification of yeasts. However, in the last decade, molecular approaches have been developed that have overcome the inherent variability of phenotypic tests. Analysis of the coenzyme Q system and the monosaccharide pattern

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of cell walls (Prillinger *et al.*, 1999), random amplification of polymorphic DNA (RAPD), microsatellite analysis (Gente *et al.*, 2002), restriction fragment length polymorphism (RFLP) of transcribed and spacer sequences of ribosomal DNA (Caggia *et al.*, 2001), chromosome polymorphism determined by pulse-field gel electrophoresis (PFGE) (Petersen and Jesperen, 2004), and sequencing of the 5.8 S rDNA or rRNA (White *et al.*, 1990), etc., have been used in the identification and classification of yeasts species. The methods of sequencing of the 5.8S rDNA/RNA as summarized and discussed by Walker (1984) are now a common practice. The procedures used for sequencing large and small subunit RNAs and DNAs are based on the dideoxy method of Sanger *et al.* (1977). Lane *et al.* (1985) have described the application of this method to rRNA sequencing through the use of oligonucleotide primers and reverse transcriptase. Most of the initial comparisons of yeast and other microorganisms were based on reverse transcriptase mediated sequencing of rRNAs because of the relative simplicity of this method over earlier rDNA sequencing techniques. Complete sequences were not often determined because McCarroll *et al.* (1983) and Lane *et al.* (1985) have demonstrated that partial sequences of small subunit rRNAs provided essentially the same phylogenies as complete sequences. White *et al.* (1990) and Kaltenboeck *et al.* (1992) have provided protocols for sequencing rDNA using specific oligonucleotide primers and the polymerase chain reaction (PCR). rDNA sequencing results in fewer artifacts than rRNA sequencing, and the method offers the opportunity to sequence both strands of the rDNA genes, thus further reducing errors. More recently, the methodologies have evolved with the introduction of automated sequencers.

3.7 Chilli

Chilli (*Capsicum* sp.) is one of the most widely used spices in foods in the world. From the various colours to the various tastes, chillies are an important spice commodity and an integral part of many cuisines. Chillies originated in the Mexico and Central America regions. Nowadays, however, every country in the world produces chilli. Globally, chilli production exceeds 14 million metric tons (Kelley and Boyhan, 2006). Fresh market production is a large part of the U.S. market, although processed chillies are common in all parts of the world as dried, pickled or otherwise processed products (Nonnecke, 1989).

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All chillies are members of the *Solanacea* family, which also includes tomato, tobacco, eggplant and Irish potato. There has been much debate over the years as to how many species of *Capsicum* truly exist. The number has fluctuated over the centuries from 1 to 90. Currently five species are recognized as domesticated, i.e., *C. annum*, *C. chinense*, *C. frutescens*, *C. pubescens*, and *C. battacum* (Armstrong, 1995). *C. annum* includes the bulk of cultivated types including bell, yellow wax, cherry, ancho, cayenne, jalapeno and serrano. *C. chinense* includes the habaneros and Scotch bonnet. Tobasco is the most notable variety in the *C. frutescens* species. The only important variety in the *C. battacum* species is the Yellow Peruvian Chilli. *C. pubescens* includes 'manzano' and 'peron' pod types. The classification of species will obviously continue to evolve in the future. There are an additional twenty or more species of wild types.

Chilli production has increased worldwide in recent years. This may be in part due to its high nutritional value. One medium green bell chilli can provide up to 8% of the Recommended Daily Allowance of Vitamin A, 18% of Vitamin C, 2% of calcium and 2% of iron. In addition, chilli contains significant amounts of vitamin B. A phenolic compound related to vanillin, called capsaicin is responsible for the pungency in chillies. Capsaicin is not spread evenly in all parts of the fruit, and various cultivars differ markedly in its content. Chilli is considered a self pollinating crop although some out crossing will occur. Although grown as an annual crop due to its sensitivity to frost, chilli is actually an herbaceous perennial and will survive and yield for several years in tropical climates (Berke *et al.*, 2002).

Hand-harvested chilli fruits in polyethylene picking buckets are usually carried to flat-bed trailers where the contents are dumped into open bulk bins for transport to a centralized packinghouse. Good harvesting management is essential for high quality chillies. Care must be taken in removing fruits from the plant with stems attached, since chillies with intact stems are more resistant to bacterial soft rot than those with torn or partial stems. Chilli showing signs of decay should not be placed in the picking buckets since it can serve as a source of inoculum to infect otherwise healthy chillies.

The most commonly used sanitizer for washing fresh chillies is certain form of chlorine, e.g. chlorine water. The concentration of chlorine in wash/cooling water

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should be held between 75 and 200 ppm “free” chlorine, depending on the product and the organic load (matter floating or suspended in the water) at the time of use. Chlorination can be accomplished by several methods, i.e. using a gas injection system, adding sodium hypochlorite, or dissolving calcium hypochlorite tablets. Since pH also has a drastic effect on chlorine’s ability to disinfect, the pH of wash/cooling water should be at 6.0-7.5 to reduce the amount of chlorine needed to maintain the recommended free chlorine levels. Excessive use of chlorine causes “gassing off” (objectionable chlorine odor) which not only irritate workers’ skin, but also corrodes equipment, and increases sanitation costs. Both chlorine and pH measurements must be documented on a quality control form in order to comply with third party audits (Hurst, 2005). The other chemicals used as sanitizers for postharvest chillies are peroxyacetic acid, ozone, bromine and iodine compounds.

Peroxyacetic acid (CH_3CO_3H) has been used in treatement of fruit and vegetable surfaces including in chilli fruit, in the formulation of 11% hydrogen peroxide and 15% peroxyacetic acid. The labeled rate for surface contact on product is 80 ppm. After the application of peroxyacetic acid, product must be rinsed with potable water. Peroxy acetic acid is a colourless liquid with an acrid odor. As a concentrate, it is considered a hazardous substance and caused severe irritant upon breathing (Suslow, 1997).

Ozone (O_3) has been used to preserve various type of foods (Haraguchi *et al.*, 1969) and reduction of fungal pathogens in fresh fruits and vegetables. Concentrations in the range of 2-3 ppm for fruits greatly reduce microbial populations. Because of its instability, ozone must be generated at the usage site. (Gammon and Kerelak, 1973). The lethal effect of ozone is a consequence of its strong oxidizing power. Extension of shelflife of chillies can be achieved by treatment with ozonated water. In addition to the antimicrobial effects of ozone, oxidation of ethylene also occurs, thus retarding metabolic processes associated with ripening. The use of ozonated wash and flume-waters in fruit and vegetable handling and processing operations provides a method to control build-up of microbial numbers, particularly in recycled water, and deserves consideration for routine use as a disinfectant for fruits and vegetables.

Bromine sanitizer for postharvest fruits and vegetables has had limited use either alone or in combination with chlorine compounds in water treatment

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programmes, but little is known about its effectiveness as a disinfectant for fruits and vegetables. Free bromine (200 ppm) does not kill *P. aeruginosa* within 15 minutes at 24°C, but does kill *E. coli, Salmonella* Typhosa and *Staphylococcus aureus* (Gershenfeld and Witlin, 1949). Others (Kristofferson, 1958; Shere *et al.*, 1962) have observed that the addition of bromine to solutions containing chlorine compounds increases antimicrobial activity, the effectiveness sometimes being synergistic.

Iodine compounds can also be used for sanitizing fruit and vegetable surfaces. Under most conditions, free elemental iodine and hypoiodous acid are believed to be the active antimicrobial agents (Odlaug, 1981). The major iodine formulations are ethanol-iodine solutions, aqueous iodine solutions and iodophors, which are combinations of elemental iodine and nonionic surfactants such as nonyl-phenol ethoxylates (Bartlett and Schmidt, 1957) or a carrier such as polyvinylpyrrolidone (Lacey, 1979). Iodophors have greater solubility in water, are less volatile and irritating to the skin than ethanolic or aqueous solutions of iodine (Lawrence *et al.*, 1957), and have a broad spectrum of activity, including yeasts and moulds. Although iodophors are minimally affected by organic matter, they may stain equipment used to handle fruits and vegetables and react with starch to form a blue-purple colour. Thus, the use of iodophors for direct-contact disinfection of many fruits and vegetables has limited potential.

Diseases of chilli are caused by fungi, bacteria, nematodes, and viruses. Diseases affect all parts of the chilli plant including the foliage, stems, roots, fruits, and young seedlings. Fungi and bacteria cause a variety of symptoms such as leaf and fruit spotting, wilting and plant death. Typical symptoms of viral infections are stunting, mottling of foliage and fruit, and small, misshapen fruit. Management of diseases should be done prior to planting of the crop and many techniques have been used to minimize losses due to disease problems. Control methods include, but are not limited to, the use of cultivars with resistance to diseases, pathogen-free seeds, sterilized equipment for transplant production, rouging and eradication of diseased plants and alternative hosts, field sites that are pathogen-free and isolated from other solanaceous crops, soil fumigation, and application of chemical pesticides and other disease-suppression compounds (microbial agents, plant materials, and other natural enemies). The control strategies using non-chemical agents are more acceptable by the

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general public (Teng, 1993). Nowadays, there are some microbial agents successfully control pests in chilli plant, i.e., *Bacillus thuringiensis* for Caterpillar control, *Verticillium lecanii* against Aphids and whitefly (Assured of Food Standard, 2007). However, the researches for control chilli diseases with microbial agents have been rarely founded, i.e. using of *Trichoderma* spp. against *Colletotrichum gloeosporioides* has been investigated, recently (Jebessa and Ranamukhaarachchi, 2006).

3.8 Chilli anthracnose

On a worldwide basis, anthracnose is the single greatest economic constraint to chili and sweet chilli production. This damaging is especially importance in chilli crops grown in tropical and subtropical areas of the world where it causes extensive pre- and postharvest losses. *Colletotrichum capsici* is a major pathogen that causes anthracnose disease on chilli fruits, accounting for 25 - 30% postharvest loss during storage and marketing (Lakshmesha *et al.*, 2005). Two serious symptoms in chilli anthracnose are ripe rot and die-black (Smith and Crasson, 1959).

3.8.1 Anthracnose symptoms

Fruit rot symptom usually occurs in the ripen fruits. A small black circular spot appears on the fruit skin and spreads along the long axis, thus becoming more or less elliptical. As the infection progresses, the spot turned diffuse and black, greenish or dirty grey or markedly delimited by a thick and sharp black outline enclosing a lighter black or straw colour area. Badly diseased fruits turn from normal red to straw colour. On this discoloured area numerous acervuli of the fungus may be found covered with minute, elevated, spherical black stromatic masses or sclerotia of the fungus. In the advanced stages the seeds are covered by a mat of fungal hyphae, and turn rusty in colour.

Another symptom of anthracnose is die-back; the disease causes necrosis of tender twigs from the tip backwards. The entire branch or the entire top of plant may wither away. The dead twigs are water-soaked to brown, becoming grayish white or straw colour in advanced stage of the disease. A large number of black dots (Figure 3-1) are found scattered all over the necrotic surface of the affected twigs. The necrotic area is sometimes separated from the healthy area by a dark brown to black band. Die-back usually appears after raining and then a prolonged deposition of dew on the plants. Only the top or few side branches may be killed, however, the entire

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plant can be withered in severe attacks. The partially affected plants bear fruits which are few and of low quality.

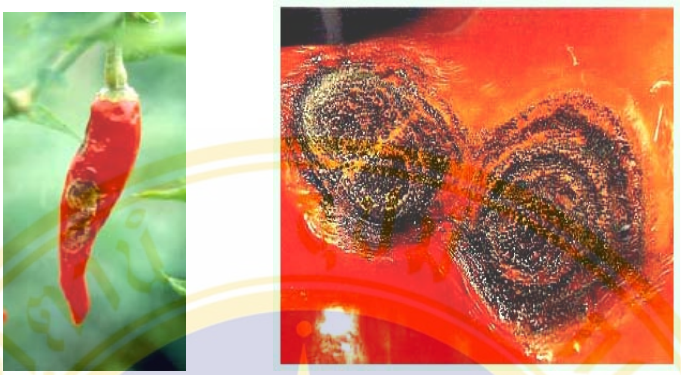

Figure 3-1 Symptoms of chilli anthracnose.

3.8.2 Causal organism of anthracnose disease (*C. capsici*)

Anthracnose is caused by several *Colletotrichum* spp. A significant causal pathogens found in Thailand are *Colletotrichum capsici*. It is also the main causal agents in tropical Asia (Chang and Chung 1985, Manandhar *et al.*, 1995), Korea (Kim *et al.*, 1989), Louisiana and Mississippi in the United State (Hadden and Black 1989; Roy *et al.*, 1997). Colony of *C. capsici* is gray or green (Figure 3-2). The mycelium of *C. capsici* is septate and inter-and intracellular. Acervulus stroma on the stem is hemispherical and 70-120 microns in diameter. Setae are scattered, dark brown, several septate, light brown at the tips, and around 150 microns in length (Figure 3-3). Conidiophores are aseptate and unbranched. Conidia in mass appear pinkish. They are borne singly at the tip of conidiophores. Individually, it is hyaline, unicellular, curved with narrow end (fusaroid) with dimension of 17-28 × 3-4 microns. Conidia germinate in water within four hours and the germ tube soon forms an appressorium. Germination of conidia of *C. capsici* is optimum at 30 ^{O}C (Misra and Mahmood, 1960). The optimum conditions for growth of the fungus are 28 ^{O}C and 92% relative humidity (Chowdhury, 1957). Maximum disease development (fruit rot) takes place at 28 ^{O}C and 95.7% relative humidity (Chowdhury, 1957). Physiology and virulence of the fungus varies with isolates indicating existence of distinct strains within the species (Misra and Dutta, 1963). The same fungus has been reported to cause leaf spots and blight of marigold, pothos, coriander, and tumeric (Hadden and

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Black, 1989). The fungus also produces a thermostable toxin that causes toxicity to host cell protoplasm without affecting the cell-wall (Narain and Das, 1970).

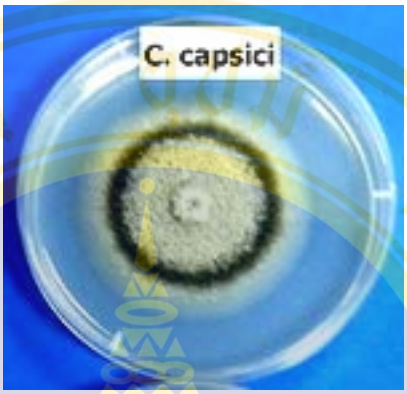
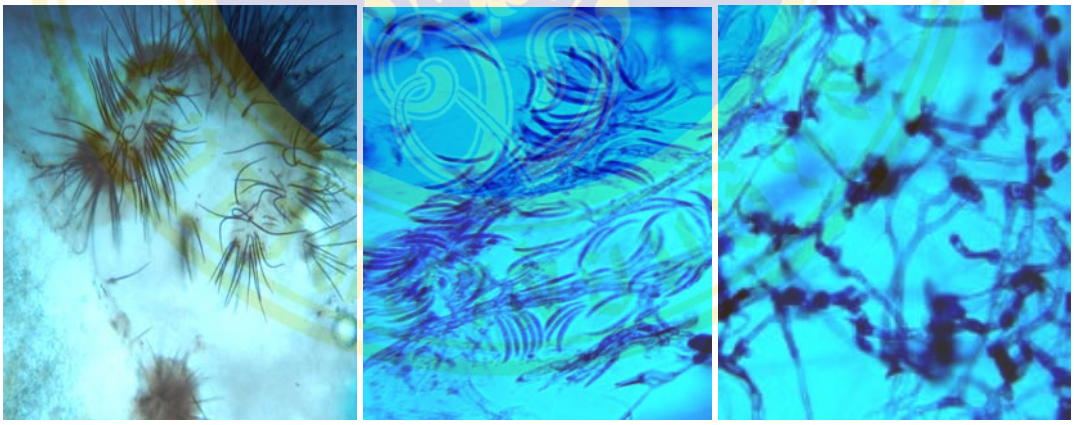


Figure 3-2 *C. capsici* colony on potato dextrose agar plate.



a b c

Figure 3-3 *C. capsici* mycelial morphology (1200×): a) part of acervuli, b) curved conidia, c) appressoria formation from hyphae.

3.8.3 The disease cycle

The fungus survives in the field in plant debris and the secondary spread takes place by wind-borne conidia. Seeds from badly diseased fruits also carry the primary inoculum. In the case of chilli fruits buried in the soil and subjected to weathering in the field, conidial germination is reduced from 96 to 30-38% at the end

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of ten months. The mycelium also remains viable in the diseased tissues of the whole fruits kept under different conditions. The seeds from the diseased fruits also carry the infection. Narain and Panigrahi (1971) have analysed the pectin content of the chilli fruits at different stages of ripening and found both qualitative and quantitative differences in carbohydrates. The ripen fruits have higher quantity of sucrose than the green fruits in addition to the presence of glucose and fructose, whereas in the green stage, only glucose and sucrose are present. An examination of the suitability of these sugars, in the presence of pectin, for the production of pectic enzymes by *C. capsici* revealed the superiority of fructose; the presence of glucose markedly reduced pectic enzyme production (Mehrotra and Aggarwal, 2003).

3.8.4 Disease management

The control of chilli anthracnose fruits rot, for many years, has relied on chemicals thus resulting in many undesirable problems. There is a need to incorporate alternative control components which are effective at the farm level. Biological control of fruit rot and die-back of chilli with plant products have been tested in many laboratories and field trials. The results show that the crude extract from rhizome, leaves and creeping branches of sweetflag (*Acorus calamus* L.), palmorosa (*Cymbopogon martinii*) oil, *Ocimum sanctum* leaf extract, and neem (*Azadirachia indica*) oil restrict growth of the anthracnose fungus. Upon testing for their efficacy to reduce fruit rot of *Capsicum*, it was found that sweet flag extract in ethyl acetate showed good inhibitory effect (Jeyalakshmi and Seetharaman 1998, Korpraditskul *et al.*, 1999). Under laboratory conditions, extract from sweet flag rhizome in ethyl acetate at 2,500 ppm can substitute 500 ppm carbendazim fungicide in minimizing anthracnose disease symptoms. (Charigkapakorn, 2000). Chemical control of the fruit-rot disease has been achieved by spraying the crop with fungicides, i.e., Perenox (0.2%), Zineb (0.25%), Mancozeb (0.25%). Although fungicides are recommended for controlling anthracnose, the breeding of resistant chilli species to anthracnose is another significant aspect of disease control. Varieties of commercial hybrid chilli have been recommended for cultivation, e.g. Bengal Green, Lorai, Perennial, H-1, H4, and H-6 (Mehrotra and Aggarwal, 2003)

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Educational review

Traditional schooling continues to emphasize rote memory, achieving high score on tests over relatively trivial material and amassing information taught for its own sake. This type of schooling fails to connect to students and their lives, and it suppresses critical thinking skills because that type of thinking disrupts the teacher's authoritarian role (Nelson *et al.*, 2000). Schooling should, instead, open ideas that stimulate students to examine their own experiences and to engage in the critical examination of social problems. To develop critical thinking skills, students need, and deserve, to participate actively in the learning process, and require materials that stretch their imaginations, their pre-existing conceptions, and their intellectual capacities (Duckworth, 1987). These skills may be acquired through participation in experiential learning activities, i.e., hands-on laboratory, field work, case study, game, etc., which are active process engaging learners in an experience that will have real consequences. The concept of experiential learning is very close to the constructivism. It stems from the concepts of learning by doing first attributed by Dewey (1938) and reflective practice postulated by Schon (1983). The experiential learning approach aims to encourage learners to feel learning as well as to think it, and to let them "try on" new behaviors and new emotional and cognitive responses (Pfeiffer and Ballew, 1988b). Thus, the combination of critical thinking through experiential learning way may be valuable for learning outcomes of students (Travis *et al*, 2004). As to the question whether the learners have achieved their learning outcomes, assessment tools play an important roles. To assess learning outcomes, multiple assessment instruments and procedures are imperative when determining student's conceptual understanding of science and when making decisions about instruction. However, effective science teacher should not only assess conceptual understanding but also thinking skills, work habits, and student's attitudes toward science and scientific processes.

3.9 Experiential learning

Experiential learning is a process whereby knowledge is created through the transformation of experience. Kolb (1984) has stated that education is becoming more accessible to all people and there has been a corresponding need for curricula to translate abstract ideas into concrete practical realities in peoples' lives. The experiential learning theory combines experience, perception, cognition, and behavior

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(Kolb, 1984). Experiential learning revolves around any experience that can be incorporated into the curriculum or program, which is complemented by a debriefing or reflection component. The latter application of experiential learning should take place in a structured environment and it becomes critical if the experience is to be transferred into higher order cognitive levels (Stone, 1994). Halpern (2003) has stated that experiential teaching and increased usage of critical thinking skills may increase the cognitive ability and retention of information of these students. The increase of these critical thinking skills allows the student to obtain, retain, and process information more efficiently (Ngeow and Kong, 2001).

John Dewey (1938) has been regarded as the most influential 20th century educational theorist who emphasize the importance of experiential learning (Kolb, 1984). Since then numerous models of experiential learning have been initiated. David Kolb and Fry (1975) have constructed a model with four modes, i.e., concrete experience, observation and reflection, the formation of abstract concepts and testing in new situations. These four modes deal with the processes whereby knowledge is gained through experience. They have represented these in the famous experiential learning circle (Figure 3-4). However, they has suggested that the learning cycle may begin at any one of the four modes, but it should remain a continuous cycle. The concrete experience results in the creation of concepts that integrate the learner's observations into logically sound theories. The learner uses these theories to make decisions and solve problems. After decisions are made and problems are solved, the learner utilizes the hands-on component of learning and applies his/her experiences.

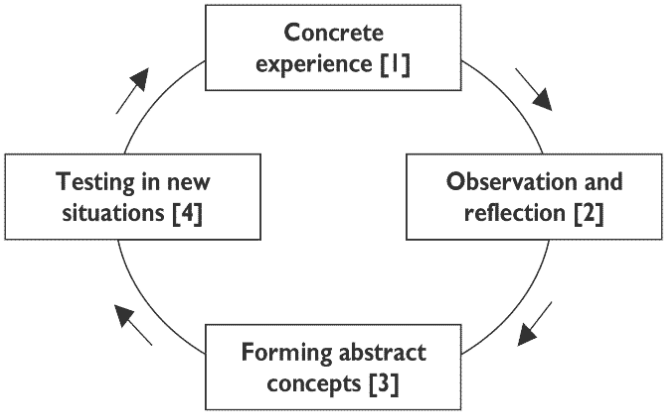

Figure 3-4 Experiential learning cycle (Kolb and Fry, 1975).

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Experiential learning programs in the sciences, although encompassing a broad spectrum of activities, can generally be grouped into two models, i.e., simulations and authentic involvement (Tuss, 1996). Following the classical education procedures used for laboratory classes, simulation model encompass contrived situations that are carefully designed to meet selected learning objectives. Traditional simulations tend to be classroom or laboratory based and regularly has scheduled class periods, instructor-supervised activities, and individual and group assignment. On the contrary, simulated experiential learning activities include (i) experimental laboratory investigations focusing on a particular apparatus, system, or instrumentation, (ii) instructor-guided explorations in which students conduct and explicitly designed study and develop a specifically targeted outcome, (iii) case studies involving field trips; classroom visits by practicing scientists, or role-play activities wherein students are placed in a work situation similar to that experienced by scientists or engineers, and (iv) real life-games; interactive participation in continued simulations of real situations. In authentic involvement models, students are exposed to real situations involving a real need to obtain a solution that has yet to be determined. Because problem-solving strategies are self-determined and self-paced by the students, the role of the supervising faculty, if any, is more advisory than instructional. The involvement is consequently more like a research scientist's or engineer's work pattern than a student's work pattern. There are many models of authentic involvement activities including (i) cooperative education and similar work-experience program, (ii) internships and apprenticeship programs involving direct supervision of a practitioner in the discipline, (iii) consulting activities involving campus-based, faculty-supervised, problem-solving experience in which problems are solicited from an outside client, and (iv) clinic or design centres administered by faculty or funded by schools.

Science is essentially a way of giving meaning and structure to our world. For students to construct and authentic view of science, a greater emphasis should be placed on the development of an experiential basis rather than the formal development of scientific facts and ideas (Martin *et al.*, 1990). Although different kinds of experiences may be required to meet the various goals of science education (Hodson, 1988), success in each one of them requires a realistic perspective of not only the content but also the processes and purposes of science, and realistic perspective

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depends on collaborative involvement of the scientific community in universities, industries, and national laboratories.

3.10 Constructivism

Constructivism is connected with experiential learning and builds on some of the ideas of Jean Piaget. In the view of constructivism, learning is a constructive process in which the learner is building an internal illustration of knowledge, a personal interpretation of experience (Chapman, 1988). This representation is continually open to modification, its structure and linkages forming the ground to which other knowledge structures are attached. Learning is an active process in which meaning is accomplished on the basis of experience. This view of knowledge does not necessarily reject the existence of the real world, and agrees that reality places constrains on the concepts that are, but contends that all we know of the world are human interpretations of our experience of the world. Conceptual growth comes from the sharing of various perspectives and the simultaneous changing of our internal representations in response to those perspectives as well as through cumulative experience (Bednar *et al.*, 1995).

The fundamental challenge of constructivism is in its changing the locus of control over learning from the teacher to the student. Educational technologists, with their foundations in behavioral psychology, have sought to design programs in such a way that students would be enticed to achieve prespecified objectives. Constructivists have said that this violates both what we know now about the nature of learning (situated, interactive) and about the nature of knowledge (perspectival, conventional, tentative, and evolutionary). They have claimed that objectives should be negotiated with students based on their own felt needs, that programmed activities should emerge from within the contexts of their lived worlds that students should work together with peers in the social construction of personally significant meaning and that evaluation should be a personalized ongoing, shared analysis of progress (Jones, 1995).

Agreeing with this view of knowledge, learning must be placed in a rich context, reflective of real world context, for this constructive process to happen and transfer to environments beyond the school or training classroom. Learning through cognitive apprenticeship, mirroring the collaboration of real world problem solving, and using the tools available in problem solving situations, are keys. How effectual or

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instrumental the learner's knowledge structure is in facilitating thinking in the content field is the measure of learning (Bednar *et al.*, 1995).

A number of constructivist models emerged over the last thirty years suggested that teachers should sequence instruction into a series of teaching/learning phases. One example of this sequence has been described as learning cycles that is closely related to experiential learning model of Kolb (1984). The learning cycle model of instruction, originally proposed by Robert Karplus (1977), is based on Piagetian theory and involves a constructivist approach to teaching (Figure 3-5). It is intended to help students progress from concrete to abstract thinking about content (i.e., from concrete to formal operations) Learning cycles teach science in three consecutive phases known as exploration, term introduction, and concept application that are based on the way people spontaneously learn about the world (Lawson, *et al.*, 1989). These phases have been described as follows: "Exploration" allow students to investigate new materials and/or ideas so that patterns of regularity can be discovered and questions are raised that students then attempt to answer. "Term introduction" allows the teacher to introduce terms to label the patterns and to explain the newly invented concepts. "Concept application" provokes students to seek the patterns elsewhere and to apply the new concepts to additional examples, often employing abstraction or generalization techniques (Lawson, 1988). Research has supported the effectiveness of the learning cycle in encouraging students to think creatively and critically, as well as in facilitating a better understanding of scientific concepts, developing positive attitudes toward science, improving science process skills, and cultivating advanced reasoning skills (Lawson, 1995).

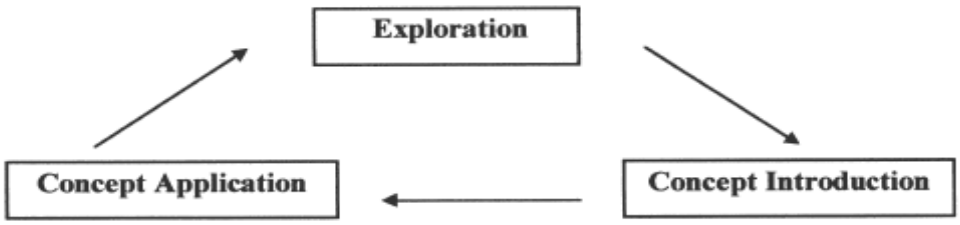

Figure 3-5 Karplus's learning cycle (Karplus, 1977).

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3.11 Hands-on laboratory in science education

Hands-on laboratory have long had a distinctive and central role in the science curriculum and science educators have suggested that many benefits accrue from engaging students in science laboratory activities (Lunetta *et al.*, 2007). At the beginning of the twenty-first century we are entering a new era of reform in science education. Both the content and pedagogy of science learning and teaching are being scrutinized, and new standards intended to shape and rejuvenate science education are emerging (National Research Council [NRC], 1996; 2000). The National Science Education Standards (NRC, 1996) and also the 2061 project (American Association for the Advancement of Science, 1990) reaffirm the conviction that inquiry in general and inquiry in the context of practical work in science education is central to the achievement of scientific literacy. Inquiry-type laboratories have the potential to develop students' abilities and skills such as: posing scientifically oriented questions (Krajcik *et al.*, 2001; Hofstein *et al.*, 2005), forming hypotheses, designing and conducting scientific investigations, formulating and revising scientific explanations, and communicating and defending scientific arguments.

Over the years, many have argued that science cannot be meaningful to students without worthwhile practical experiences in the school laboratory. Unfortunately, the terms school laboratory or lab and practical have been used, too often without precise definition, to embrace a wide array of activities. Typically, the terms have meant experiences in school settings where students interact with materials to observe and understand the natural world. Some laboratory activities have been designed and conducted to engage students individually, while others have sought to engage students in small groups and in large-group demonstration settings. Teacher guidance and instructions have ranged from highly structured and teacher-centreed to open inquiry. The terms have sometimes been used to include investigations or projects that are pursued for several weeks, sometimes outside the school, while on other occasions they have referred to experiences lasting 20 minutes or less. Sometimes laboratory activities have incorporated a high level of instrumentation, and at other times the use of any instrumentation has been meticulously avoided.

Many research studies have been conducted to investigate the educational effectiveness of laboratory work in science education in facilitating the attainment of

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the cognitive, affective, and practical goals. These studies have been critically and extensively reviewed in the literature (Hofstein and Lunetta, 2004; Hodson, 1993). From these reviews it is clear that in general, although the science laboratory has been given a distinctive role in science education, research has failed to show simple relationships between experiences in the laboratory and student learning. Hodson (1990) has criticized traditional laboratory work and claimed that it is unproductive, and confusing, since it is very often used without any clearly thought-out purpose, and he called for more emphasis on what students are actually doing in the laboratory. Tobin (1990) wrote that: “Laboratory activities appeal as a way to learn with understanding and, at the same time, engage in a process of constructing knowledge by doing science”. He has also suggested that meaningful learning is possible in the laboratory if students are given opportunities to manipulate equipment and materials in order to be able to construct their knowledge of phenomena and related scientific concepts. According to Gunstone (1991), using the laboratory to have students restructure their knowledge may seem reasonable but this idea is also naive since developing scientific ideas from practical experiences is a very complex process. Gunstone and Champagne (1990) suggested that meaningful learning in the laboratory would occur if students were given sufficient time and opportunities for interaction and reflection. Gunstone and Champagne have stated that students generally do not have time or opportunity to interact and reflect on central ideas in the laboratory since they are usually involved in technical activities with few opportunities to express their interpretation and beliefs about the meaning of their inquiry. In other words, they normally have few opportunities for metacognitive activities. Bird (1990) has suggested that these metacognitive skills are "learning outcomes associated with certain actions taken consciously by the learner during a specific learning episode." Metacognition involves elaboration and application of one's learning, which can result in enhanced understanding. According to Gunstone and Champagne (1990), the challenge is to help learners take control of their own learning in the search for understanding. In the process, it is vital to provide opportunities that encourage learners to ask questions, suggest hypotheses, and design investigations, i.e., minds-on as well as hands-on. There is a need to provide students with frequent opportunities for feedback, reflection, and modification of their ideas (Barron *et al.*, 1998). As Tobin

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(1990) and Polman (1999) have noted, in general, research has not provided evidence that such opportunities exist in most schools in the United States, or, for that matter, in other countries.

A constructivist model currently serves as a theoretical organizer for many science educators who are trying to understand cognition in science (Lunetta, 1998), i.e., learners construct their ideas and understanding on the basis of a series of personal experiences. Learning is active, interpretive, iterative process (Tobin, 1990). Moreover, there is a growing sense that learning is contextualized and that learners construct knowledge by solving genuine and meaningful problems (William and Hmelo, 1998). Experiences in the school laboratory can provide such opportunities for students if the expectations of the teacher enable them to engage intellectually with meaningful investigative experiences upon which they can construct scientific concepts within a community of learners in their classroom (Penner *et al.*, 1998). A social constructivist framework has special potential for guiding teaching in the laboratory. Millar and Driver (1987) are among those who recommended the use of extended, reflective investigations to promote the construction of more meaningful scientific concepts based upon the unique knowledge brought to the science classroom by individual learners. An assumption is that when students interact with problems they perceive to be meaningful and connected to their experiences, and when teachers are guided by what we know about learning, the students can begin to develop more scientific concepts in dialogue with peer investigators (Hofstein and Lunetta, 2004).

3.12 Conceptual understanding

Concepts are categories into which experiences are organized and the larger network of intellectual relationships brought about through categorization (Cooper, 1986; Roe *et al.*, 1987). Understanding a concept requires some level of critical thinking in order to make associations between words and ideas according to certain criteria. While on the surface it appears as though things in our world form natural groups and categories, concepts are simply the names that people learn to associate with them. This summary of concepts does not address the fact that concepts can be false, and neglects that the human mind may impose as well as discover it. Research in cognitive psychology has suggested that concepts are more than just lists of characteristics (Glynn and Duit, 1995). An individual's knowledge and theories in the

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organization of concepts are also important. People may demonstrate a bias by assuming that categories have defining properties and then theorize about what exactly those properties are. Theories such as these may fall between the ideal properties and the actual properties, leaving the structure of that person's concepts to be imposed and discovered via the interaction of the mind and world.

To improve the conceptual understanding of science, teachers must be selective in the concepts they include in instruction. Much depends on the background that students bring to the course. This means not that more content should be moved to lower grade levels but that the National Science Education Standards (1996) should be used as a guide to provide reasonable levels of content. The standards carefully delineate what leading experts in the field deem appropriate for most students at each grade level. Increasing the content may force students to memorize and turn out to be detrimental to conceptual understanding. In the process of selecting concepts to teach, it is important to remember that helping students understand a concept involves more than getting them to provide definitions of new vocabulary words. Concepts are taught in two major ways (Yager, 1996). In deductive teaching, a definition is presented and linked to positive and negative examples. An inductive sequence presents students with examples and then asks them to analyse the examples and identify essential characteristics. Both types of concept lessons are interactive to encourage student involvement and to help the teacher gauge whether students understand the concept.

3.13 Critical thinking and science education

Critical thinking is an important and vital topic in modern education. All educators are interested in teaching critical thinking to their students. Academic staff should be informed about the strategy of teaching critical thinking skills, identify areas in one's courses as the proper place to emphasize and teach critical thinking. They should also develop and use some problems in exams that test students' critical thinking skills (Facione and American Philosophical Association, 1990). The purpose of specifically teaching critical thinking in the sciences or any other discipline is to improve the thinking skills of students and thus better prepare them to succeed in the world (Tsui, 2002).

Critical thinking can be described as the scientific method applied by ordinary people to the ordinary world (Resnick, 1987). This is true because critical

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thinking mimics the well-known method of scientific investigation: a question is identified, a hypothesis formulated, relevant data sought and gathered, the hypothesis is logically tested and evaluated, and reliable conclusions are drawn from the result. All of the skills of scientific investigation are matched by critical thinking, which is therefore nothing more than scientific method used in everyday life rather than in specifically scientific disciplines or endeavors. Critical thinking is scientific thinking, many books and papers describing critical thinking present its goals and methods as identical or similar to the goals and methods of science. A scientifically-literate person, such as a math or science instructor, has learned to think critically to achieve that level of scientific awareness. However, any individual with an advanced degree in any university discipline has almost certainly learned the techniques of critical thinking.

Critical thinking is also critical inquiry, so such critical thinkers investigate problems, ask questions, pose new answers that challenge the status quo, discover new information that can be used for good or ill, question authorities and traditional beliefs, challenge received dogmas and doctrines, and often end up possessing power in society greater than their numbers (Tsui, 1998). It may be that a workable society or culture can tolerate only a small number of critical thinkers, that learning, internalizing, and practicing scientific and critical thinking is discouraged. Most people are followers of authority: most do not question, are not curious, and do not challenge authority figures who claim special knowledge or insight. Most people, therefore, do not think for themselves, but rely on others to think for them. Most people indulge in wishful, hopeful, and emotional thinking, believing that what they believe is true because they wish it, hope it, or feel it to be true. Most people, therefore, do not think critically.

3.14 The core of critical thinking

Critical thinking is a higher-order thinking skill which mainly consists of evaluating arguments. It is a purposeful, self-regulatory judgment which results in interpretation, analysis, evaluation, and inference, as well as explanations of the evidential, conceptual, methodological, or contextual considerations upon which the judgment is based (Astleitner, 2002). Facione (2006) has characterized and defined the

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core of critical thinking which consisted of interpretation, analysis, evaluation, inference, explanation, and self-regulation as shown in Figure 3-6.

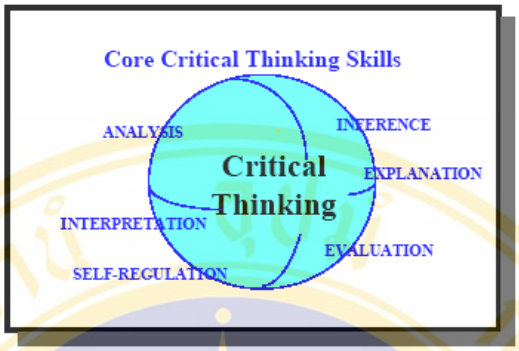

Figure 3-6 Core of critical thinking skills (Facione, 2006).

3.14.1 Interpretation

Interpretation is to comprehend and express the meaning or significance of a wide variety of experiences, situations, data, events, judgments, conventions, beliefs, rules, procedures, or criteria. Interpretation includes the sub-skills of categorization, decoding significance, and clarifying meaning.

3.14.2 Analysis

Analysis is to identify the intended and actual inferential relationships among statements, questions, concepts, descriptions, or other forms of representation intended to express belief, judgment, experiences, reasons, information, or opinions. The experts include examining ideas, detecting arguments, and analyzing arguments as sub-skills of analysis.

3.14.3 Evaluation

Evaluation means to assess the credibility of statements or other representations which are accounts or descriptions of a person's perception, experience, situation, judgment, belief or opinion, and to assess the logical strength of the actual or intended inferential relationships among statements, descriptions, questions or other forms of representation.

3.14.4 Inference

Inference means to identify and secure elements needed to draw reasonable conclusions, to form conjectures and hypotheses, to consider relevant information, and to educe the consequences flowing from data, statements, principles,

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evidence, judgments, beliefs, opinions, concepts, descriptions, questions, or other forms of representation. As sub-skills of inference the experts list querying evidence, conjecturing alternatives, and drawing conclusions.

3.14.5 Explanation

Explanation means as being able to present in a cogent and coherent way the results of one's reasoning. This means to be able to give someone a full look at the big picture: both to state and to justify that reasoning in terms of the evidential, conceptual, methodological, criteriological, and contextual considerations upon which one's results were based; and to present one's reasoning in the form of cogent arguments. The sub-skills under explanation are describing methods and results, justifying procedures, proposing and defending with good reasons, one's causal and conceptual explanations of events or points of view, and presenting full and well-reasoned, arguments in the context of seeking the best understandings possible.

3.14.6 Self-regulation

Self-regulation means self-consciously to monitor one's cognitive activities, the elements used in those activities, and the results educed, particularly by applying skills in analysis, and evaluation to one's own inferential judgments with a view toward questioning, confirming, validating, or correcting either one's reasoning or one's results. The two sub-skills here are self-examination and self-correction.

3.15 Classroom assessments

Classroom assessment is a simple method that instructors can use to collect feedback, early and often, on how well their students are learning what they are being taught. The purpose of classroom assessment is to provide faculty and students with information and insights needed to improve teaching effectiveness and learning quality. Instructors use feedback gleaned through classroom assessment to inform adjustments in their teaching. Instructor also shares feedback with students, using it to help them improve their learning strategies and study habits in order to become more independent, successful learners. The standard assessment techniques used in science education are summarized as follows:

3.15.1 Assessment of critical thinking skills

Assessment of critical thinking skills can use the students' responses from the activities that promote critical thinking to assess whether they are, indeed,

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reaching your critical thinking goals. It is important to establish clear criteria for evaluating critical thinking. Even though many of us may be able to identify critical thinking when we see it, explicitly stated criteria help both students and teachers know the goal toward which they are working. An effective criterion measures which skills are present, to what extent, and which skills require further development. The following are characteristics of work that may demonstrate effective critical thinking:

1) Accurately and thoroughly interprets evidence, statements, graphics, questions, literary elements, etc
2) Asks relevant questions
3) Analyses and evaluates key information, and alternative points of view clearly and precisely
4) Fair-mindedly examines beliefs, assumptions, and opinions and weights them against facts
5) Draws insightful, reasonable conclusions
6) Justifies inferences and opinions
7) Thoughtfully addresses and evaluates major alternative points of view
8) Thoroughly explains assumptions and reasons

It is more useful to assess students throughout a course, so the instructor can see if criteria require further clarification and students can test out their understanding of the course criteria and receive feedback. This will help student to reflect on their own work and improve the quality of their thinking and writing.

3.15.2 Concept mapping

Concept mapping is a technique for visualizing the relationships among different concepts. In concept map diagram, various concepts are connected with labelled arrows, in a downward-branching hierarchical structure. The relationship between concepts is articulated in linking phrases, e.g., "gives rise to", "results in", "is required by," or "contributes to". The technique of concept mapping was developed by Joseph D. Novak and his research team at Cornell University in the 1970s as a means of representing the emerging science knowledge of students. It is a powerful tool for linking knowledge and could be a key to developing strong performance assessments that ought to be designed to generate both an assessment of how students are applying

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concepts and to assess the deep understanding that students are gaining. Concept mapping in the science classroom, particularly for biology instruction, improves science achievement and attitudes. The use of concept maps appears to be more beneficial at the end of a unit than at the beginning. Although there appears to be no difference in student achievement whether the maps are constructed by the teacher or by the students, there are greater gains in achievement when students supply the key terms to construct the maps (Lawson, 1994).

Anderson-Inman and Zeitz (1993) have cited considerable evidences that concept mapping promotes meaningful learning in science. In science education, concept mapping has been widely recommended and used in a variety of ways to observe change in students' understanding of concepts over time, to assess what the learner knows, and to reveal their unique thought processes. It has been used in evaluation of science curriculum and instructional activities for promoting conceptual understanding and positive learner attitudes toward science (American Association for the Advancement of Science, 1990). Concept mapping can also be used as an accommodation strategy for students with learning difficulties; computer-based concept mapping assists students who are oriented toward visual learning (or who have difficulty reading and writing text) to graphically represent what they are learning (Ruiz-Primo and Shavelson, 1996). Students' graphic representation of science concepts can be tracked over time, which provides teachers with a method for monitoring conceptual growth as a function of instruction.

3.15.3 Conceptual test

Effective concept tests are not simple content-based multiple-choice questions that rely on the student re-reading their lecture notes or memorizing a fact or definition. Instead, concept tests are designed to assess student’s understanding of the principal concepts underlying the lecture material. Concept tests may be considered as equivalent to comprehension, application or analysis questions as defined by Bloom's Taxonomy (McConnell *et al.*, 2006).

Bloom’s taxonomy is to be a useful framework for developing multiple choice, short-answer, matching, and essay questions that can involve students in complex cognitive tasks. The classification of the task/item in a certain level of Bloom’s taxonomy based on the highest level of cognitive task posed to the student.

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That is, if the highest level of expectation for the student is to remember some facts, definitions, terminology, symbols, etc., such a task is at the knowledge level. If the student is expected to translate, illustrate, extrapolate, estimate, predict, identify/distinguish, and interpret-without necessarily relating it to other material or seeing its fullest implications such as task is classified at the comprehension level. A task that requires students to use abstraction and apply it in particular and concrete situations is classified at the application level. When the student is expected to break down information into its constituent parts, considering their relationships and organizational principles, the task represents the analysis level. When the situation/task is opposite to analysis, the student is expected to put together elements and parts to form a whole: it is said to be at the synthesis level. And finally, when the student is required to use criteria and judgment to justify something based on internal/external evidence, the task is at the evaluation level. (Vidakovic *et al.*, 2003). The cognitive domain of Bloom's Taxonomy (Bloom, 1956) is shown in Table 3-3.

Table 3-3 Bloom's Taxonomy

Title	Definition	Question
Knowledge	The ability to recall information	Name, list, tell, who, what, when, where
Comprehension	The ability to understand and explain information	Why, how, explain, describe, match, identify, restate
Application	The ability to use information in a new situation	Arrange, draw, dramatize, interview, sketch
Analysis	The ability to categorize information and to perceive similarities and differences	Classify, compare, contrast, graph, relate, diagram
Synthesis	The ability to create by combining more than one piece of information	Invent, design, predict, estimate
Evaluation	The ability to make judgments concerning given information, using supporting data	Support, decide, debate, choose, recommend, editorialize, determine

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In summary, Bloom's Taxonomy is a way to classify instructional activities or questions as they progress in difficulty. The lower levels require less in the way of thinking skills. As one moves down the hierarchy, the activities require higher level thinking skills (Bloom, 1956).

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CHAPTER IV
MATERIALS AND METHODS

The research is divided in 2 parts, i.e., scientific and educational parts. The former describes the use of antagonistic yeast to control anthracnose disease in postharvest chilli. Yeast antagonists of anthracnose disease caused by *C. capsici* were screened from fruits and vegetables, identified and detected for their biological control efficacies. The mode of action of the best effective yeast against *C. capsici* was investigated. The latter part deals with application of the results from the scientific part on yeast biological control to construct hands-on experiment for highschool students by using the experiential learning model. This part includes research design, assessment tools, data collection and analysis.

Scientific part

This part comprises the research materials and methods used in the experiments on screening, identifying and testing for yeast antagonists against *C. capsici*, the causative agent of chilli anthracnose. Methods used for investigating modes of action of the most effective yeast antagonist were included.

4.1 Materials

4.1.1 Chemicals

Chemicals used in this study are listed in Table 4-1.

Table 4-1 Lists of chemicals.

Chemicals	Manufacturers
1. Culture media -Yeast extract, potato dextrose agar, dextrose, malt extract, peptone, agar, and beef extract	-Difco Laboratories, USA

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Table 4-1 Lists of chemicals (continued).

Chemicals	Manufacturers
2. Enzymatic substrates	
- Colloidal chitin, colloidal chitin azure, laminarin, and laminarin azure	-Loewe Biochemica, Germany -Sigma-Aldrich, USA
3. Sugars	
-Glucose, galactose, maltose, melibiose, fructose, raffinose, lactose, and mannose	-Merck & Co., Germany
4. Dyes	
-Lactophenol cotton blue, methylene blue, and toluidine blue-O	-Merck & Co., Germany
5. Miscellaneous compounds:	
- NH_4NO_3, $NaNO_3$, $Ca(NO_3)_2{\cdot}4H_2O$, $Mg(NO_3)_2{\cdot}6H_2O$, $CaCl_2$ KNO_3, $Fe(NO_3)_3{\cdot}9H_2O$, Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, $MgSO_4$, HCl, NaOH, ethanol, methanol, Tris/HCl, $NaPO_4$, citric acid, glucose, bovine serum albumin, God-Perid glucose test kit	-Boehringer Mannheim, GMBH, Germany -Merck & Co., Germany -Sigma-Aldrich, USA

4.1.2 Sources of fruits and vegetables

Yeast antagonists of anthracnose disease caused by *C. capsici* were screened from 11 species of fruits and vegetables. They were banana (*Musa sapientum* L.), mango (*Mangifera indica* L.), longan (*Dimocarpus longan* Lour.), pineapple (*Ananus comosus* Merr.), rambutan (*Nephelium lappaceum* L.), rose apple (*Eugenia javanica* Lamk.), sapodilla (*Achras sapota* L.), bird pepper (*Capsicum frutescens* L.), chilli (*C. annuum* L. var. *acuminatum* Fingerh.), sweetbell pepper (*C. annuum* L. var. *grossum*), eggplant (*Solanum melongena* L.), and plate brush (*S. torvum* Swartz) harvested from untreated fields in Singhburi Province, in the central region of

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Thailand. Red chilli fruits (*C. annuum* L. var. *acuminatum* Fingerh.), which had not been treated with any fungicides were used for a biological control assay.

4.1.3 The fungal pathogen

C. capsici DOAC 1511 was obtained from the Mycological Laboratory of Department of Agriculture (DOA), Thailand.

4.2 Screening for yeast antagonists against *C. capsici* from fruits and vegetables

4.2.1 Yeast isolation

Epiphytic yeasts were isolated from the surface of fruits and vegetables according to the method of Assis and Mariano (1999). Ten grams of the sample were suspended in 100 ml sterile distilled water and shaken vigorously for a few minutes. Serial dilutions of the yeast sample suspension were made in sterile distilled water. An aliquot of 0.1 ml of each dilution was plated on yeast malt extract agar (YM agar) adjusted to pH 3.5 with sterile tartaric acid and the cultured plates were incubated at 28 ^{O}C for 48 h. All colonies of yeast were examined under the microscope (Accumax XSZ-107) and 225 colonies from fruits and vegetables were chosen on the basis of their different visual characteristics. The yeast isolates were re-streaked on YM agar to obtain pure cultures and they were maintained on nutrient yeast dextrose agar (NYDA) slants containing 8 g l^{-1} nutrient broth, 5 g l^{-1} yeast extract, 10 g l^{-1} glucose, and 20 g l^{-1} agar. The cultures were stored at 4 ^{O}C until further study on antagonistic activity.

4.2.2 Studies on severity of disease caused by *C. capsici*

The fungal pathogen (*C. capsici*) maintained on potato dextrose agar (PDA) slants at 4 ^{O}C was determined the ability to cause disease severity. The severity of anthracnose disease on chilli fruits caused by *C. capsici* was studied by using 30 wounded chilli fruits (one wound per fruit) inoculated with *C. capsici* at concentrations of 5×10^2, 5×10^3, 5×10^4, 5×10^5, and 5×10^6 spores ml^{-1}. Control fruits were treated with sterile distilled water. All chilli fruits were put on 17 × 1 × 4 cm plastic trays wrapped with high density polyethylene sleeve. Sterile water was used to maintain high humidity (95% relative humidity (RH)) of the fruit trays. The trays were stored in a ventilated cabinet (Memmert, type UM 600, Schwabach, Germany) in the dark at 28 ^{O}C. Lesion diameter (average length of lesion in x-axis and y-axis) was

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measured five days after inoculation. The experiments were repeated three times and each treatment was conducted in triplicate of 30 wounded control and experimental chilli fruits.

4.2.3 Screening for yeast antagonists against *C. capsici*

Screening for yeast antagonists against *C. capsici* was conducted in two steps. Firstly, 225 colonies of yeast colonies isolated from fruit and vegetable samples were streaked on potato dextrose agar (PDA) plates containing *C. capsici* at $5x10^4$ spores ml^{-1}. The cultures were incubated at 28 ^{O}C for five days. Then yeast isolates that resisted mycelial invasion (54 colonies) were chosen for the well test according to the method of He *et al.* (2003). Yeast isolates were cultured in nutrient yeast dextrose broth (NYDB) on a rotary shaker for 48 h. Yeast cells were collected by centrifugation at 3000 × *g* for 20 min and washed twice with sterile distilled water (DW) and re-suspended in DW. To test for their antagonistic activities, the washed yeast cell suspension (20 µl of 5×10^9 cells ml^{-1}) was added into a well (6 mm in diameter) in the centre of an agar plate of PDA plus 15% chilli juice and kept for 1-2 h to allow penetration of the cell suspension into the well. Then 20 µl of 5×10^4 spores ml^{-1} of *C. capsici* (optimal concentration as found from experiment in section 4.2.2) were applied to the well. All plates were incubated at 28 ^{O}C. The fungal growth was observed for each plate and diameters of fungal colonies (average length of diameter in *x*-axis and *y*-axis) were measured five days after incubation. Each treatment (each isolate) was done in triplicate and each experiment was conducted three times. The yeast isolates that completely inhibited fungal growth called yeast antagonist were used for further studies.

4.3 Identification of yeast antagonists

4.3.1 Identification of yeast antagonists by rDNA sequence comparison

Identification of antagonistic yeasts was done using the rDNA sequence comparison technique as described by White *et al.* (1990) and Mitchell *et al.* (1992). The reaction mixture contained specific primers for D1/D2 region of the large subunit, the 26S rDNA, that were NL-1(5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3′). In addition, primers of ITS region (Internal Transcribed Spacer region), ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and

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ITS4 (5′-TCCTCCGCTTATTGATATG-3′), were also used to amplify the intervening 5.8S gene for achieving a high separation value (Kurtzman, 1992; Martorell *et al.,* 2005; Suh and Blackwell, 2004). The 26S and 5.8S genes were amplified by the PCR technique, which yielded about 600 bp in each DNA fragment. Aliquots of 10 µl of amplified products were separated electrophoretically on 0.8% (w/v) agarose gel in Tris-borate-EDTA (TBE) buffer at a constant voltage of 100 V for 35 min. The bands were stained with ethidium bromide and photographed under transilluminated UV light. Then the products were purified by using QIAquick (QIAGEN, Germany). Sequencing was carried out using the Automate DNA Sequencer (3100-Avanti Genetic Analyzer). The sequences were aligned and compared with the NCBI database by the Internet using Basic Local Alignment Search Tool (NCBI BLAST: Bethesda, Maryland, USA) (Altschul *et. al*., 1997).

4.3.2 Examination of morphological and physiological characteristics of yeast antagonists

Morphological characteristics of yeast antagonists were examined by observing cell and colony patterns according to the methods described by Kurtman and Fell (1998). Yeast cells from different isolates were cultured in liquid medium (5% malt extract broth) for 2-3 days at 25 ^{O}C. The resulting yeast cells were then observed for shape and spore, i.e., whether they existed singly, paired, or aggregated in large clumps. The average length and width of 20 cells from each isolate were measured. Colony morphologies of each yeast isolate were examined in cultures grown in solid medium (5% malt extract agar) at 25 ^{O}C. The features of yeast cultures on the plates, i.e., colour, texture were recorded 3-7 days after incubation. Physiological characteristics of yeast antagonists were determined by detecting their ability to ferment certain sugars semi-anaerobically and to assimilate a variety of carbon compounds as major source carbon in aerobic condition. These tests were repeated three times for each strain. The results on morphological and physiological characteristics were used in the confirmation of yeast identification by the rDNA sequencing technique.

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4.4 Biological control efficacy of the antagonistic yeasts

Screening of yeast isolates for biological control efficacy was also done by using *in vivo* tests (Dan *et al.,* 2003). Chilli fruits (no wound or scar on the surface) from untreated field were selected for the experiments. They were surface-sterilized with 0.5% NaCl for 5 min and then washed with tap water. After air drying, chilli fruits were treated with 70% ethanol. Each fruit was wounded by using a sterile cork-borer (6 mm in diameter and 1mm in depth), one wound per fruit.

The four strains of yeast isolates with high biological control efficacies, selected based on data from the *in vitro* tests (section 4.2.3), were cultured in NYDB. The cells were collected by centrifugation at 3000 × *g* for 20 min. Yeast cells were washed twice with DW and re-suspended in DW. Then 20 µl of cell suspension of each yeast strain at concentration of 5×10^9 cells ml^{-1} was applied to the wound of 30 surface sterilized chilli fruits. After 1-2 h to allow the yeast cells to penetrate into the wound, 20 µl of *C. capsici* at a concentration of 5×10^4 spores ml^{-1} was added to the wound. Chilli fruits were put on plastic trays wrapped with polyethylene sleeve and stored in a ventilated cabinet (95% RH) in the dark at 28 ^{O}C. Percentage of disease incidence and severity of disease, as indicated by increased wound diameter (lesion diameter) were recorded five days after storage. The ability to reduce disease incidence of these yeast strains was compared. For control, wounded chilli fruits were inoculated with *C. capsici* only. There were three replicates of thirty wounded chilli fruits per treatment. The whole experiment was conducted three times.

4.5 Effects of yeast cell concentrations and storage temperatures on biological control efficacy

Thirty wounded chilli fruits were used for each experiment on the biological control efficacy of various concentrations of the four antagonistic yeast strains. The wounds were inoculated with 20 µl of each antagonistic yeast isolate at concentrations of 5×10^6, 5×10^7, 5×10^8, and 5×10^9 cells ml^{-1} as counted by a hemocytometer. After air drying for 1-2 h, 20 µl of *C. capsici* at concentrations of 5×10^4, 5×10^5, and 5×10^6 spores ml^{-1} were added to each wound. The chilli fruits were placed on plastic trays wrapped with polyethylene sleeve (95% Relative humidity) and stored in a

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ventilated cabinet in the dark at 28 ^{O}C for five days before the measurement of the disease incidence and severity.

To determine the effect of storage temperature on biological control efficacy, thirty wounded chilli fruits were inoculated with 20 µl of 5×10^8 cells ml^{-1} of yeast cell suspension. After 1-2 h, 20 µl of 5×10^4 spores ml^{-1} of *C. capsici* were applied as described above. Disease incidence was recorded five days after incubation at 18, 23, 28, and 33 ^{O}C.

As positive and negative controls for 0 and 100% infection in all of the *in vivo* tests, 20 µl of sterile distilled water and a spore suspension of *C. capsici* (20 µl of 5×10^4, 5×10^5, and 5×10^6 spores ml^{-1}) were added to the wounds. Each treatment was done in triplicate of thirty wounded chilli fruits and the entire experiment was repeated three times. The data were transformed into a percentage of biological control efficacy (BC) and disease incidence (DI) according to the formula: %BC = [(T - A)/T] × 100, where T is the number of infected wounds inoculated with *C. capsici* only, and A is the number of infected wounds inoculated with the antagonist(s) and the pathogen. The percentage of disease incidence, %DI = (A/T) × 100.

4.6 Effects of the yeast antagonists on postharvest disease control

Intact chilli fruits (no wound or scar on the surface) from untreated orchards were cleaned with tap water. They were tested for preservation by the four antagonistic yeast strains compared to that by water treatment and chemical treatment. Each group of thirty fruit samples were thoroughly sprayed with 300 ml of 5×10^8 cells ml^{-1} of each yeast cell suspension in distilled water. The other two groups were sprayed with 300 ml of distilled water, and chlorinated water (200 ppm chlorine), respectively. Fruits were air dried, and placed on plastic trays wrapped with polyethylene sleeve. All samples were kept in the dark at 28 ^{O}C for 1-2 h followed by storage at 10 ^{O}C (95% Relative humidity) for 45 days. The percentage of disease incidence in each group was recorded at different time intervals (15, 30, and 45 days). Each treatment was performed in triplicate of 30 wounded chilli fruits and the entire experiment was conducted three times

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4.7 Modes of action of yeast antagonist, strain R13 on *C. capsici* infected chilli fruits

Yeast *P. guilliermondii* strain R13 isolated from rambutan fruit (*Nephelium lappaceum* L.) has the highest biological control efficacy for chilli anthracnose caused by *C. capsici* (results from section 4.4-4.6). Modes of action between the yeast antagonist and the fungal pathogen investigated in this study included nutrient competitions, secretion of hydrolytic enzymes and killer toxin production.

4.7.1 Effects of yeast *P. guilliermondii* strain R13 on protection of chilli tissues from *C. capsici* infection

To investigate for the biological control capability of *P. guilliermondii* strain R13 on anthracnose disease, the effects of inoculated yeast cells and fungal pathogen on tissues of wounded chilli fruits were observed. Chilli fruits were wounded (6 mm in diameter) with sterile cork borer and treated with 20 µl of cell suspensions of *P. guilliermondii* (5×10^8 cells ml^{-1}) or sterile distilled water for control. They were then inoculated with 20 µl of spore suspension of *C. capsici* (5×10^4 spores ml^{-1}), and kept for 5 days in a ventilated incubator in the dark at 28 ^{O}C. The chilli fruit tissues were removed from centre of the infected areas using a razor blade. Thin sections of the tissue were mounted on glass slides, stained with 1.0% toluidine blue-O for 10 min, rinsed with deionized water, and examined under the light microscope equipped with a camera. The experiment was performed in triplicate with thirty fruits each treatment.

4.7.2 Studies on the interaction between *P. guilliermondii* strain R13 and *C. capsici* on the surface of wounded chilli fruits

The interaction between *P. guilliermondii* strain R13 *and C. capsici* was investigated on the surface of wounded chilli. Chilli fruits were wounded using the sterile cork borer as described in section 2.2. Each wound was inoculated with 20 µl of spore suspension of *C. capsici* (5×10^4 spores ml^{-1}) and cell suspension of yeast strain R13 (5×10^8 cells ml^{-1}) and then overlaid of a piece of sterile cellophane disk. In another set of experiment, the inoculated *C. capsici* spores (5×10^4 spores ml^{-1}) on the wound were allowed to germinate and grow for 36 h on the chilli wound before applying 20 µl of the yeast cells (5×10^8 cells ml^{-1}) to the margin of *C. capsici* hyphae and overlaying with a piece of sterile cellophane disk. All fruits were incubated at 28

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^{O}C on enclosed plastic trays for an additional 24 h in a ventilated cabinet (95% relative humidity) in the dark. Small sections of the cellophane disk were rinsed with distilled water containing 1% (v/v) Tween 20. They were then observed for interaction between the yeast strain R13 and *C. capsici* under light microscope. As a negative control for unattachment to hypha of *C. capsici*, *Issatchenkia orientalis* strain ER1, yeast strain isolated from eggplant was used. The experiment was performed in triplicate with 30 fruits each treatment.

4.7.3 Studies on the role of competition for nutrients

The role of competition for nutrients on the biological control capability of *P. guilliermondii* strain R13 was investigated. Strain 13 (50 µl at 5×10^8 cells ml^{-1}) and *C. capsici* (50 µl at 5×10^4 spores ml^{-1}) were added to test tubes containing 5 ml of chilli juice supplemented with sugars or nitrates. The sugars, which were used at concentrations of 0.05-2.00% (w/v), were glucose, sucrose, and fructose. The nitrates, which were used at a concentration of 0.1%, were NH_4NO_3, $NaNO_3$, $Ca(NO_3)_2{\cdot}4H_2O$, $Mg(NO_3)_2{\cdot}6H_2O$, KNO_3, and $Fe(NO_3)_3{\cdot}9H_2O$. For controls, either *C. capsici* alone or *C. capsici* plus yeast strain R13 were added to the 5 ml of chilli juice in test tubes lacking supplemental sugars or nitrates. In another control, *C. capsici* alone was added to chilli juice with supplemental sugars or nitrates. For controls on effects of nutrients on yeast, only yeast strain R13 was added to chilli juice, both in the presence and absence of sugars or nitrates. After 16 h at 28 ^{O}C, a few drops from each tube were placed in a haemacytometer, and at least 100 *C. capsici* spores per treatment were observed at 100x with a light microscope; germination (±) and germ tube length were determined for each spore. There were three replicate tubes per treatment, and the experiment was performed three times

4.7.4 Qualitative detection of hydrolytic enzyme activity from *P. guilliermondii* strain R13

Activities of chitinase and β-1,3-glucanase from *P. guilliermondii* strain R13 were detected qualitatively in agar plate according to Strauss *et al.* (2001) with some modifications. Yeast strain R13 was grown on YPD plate containing 1% yeast extract, 2% peptone, 2 % glucose, and 2% agar for 2 days at 28 ^{O}C before used for enzyme detection.

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4.7.4.1 Qualitative detection of chitinase activity in agar plate

The chitinase detection agar plates were prepared according to the method of O'Brien and Colwell (1987). The bottom layer contained 15 ml of basal medium (0.65 g l^{-1} Na_2HPO_4, 1.5 g l^{-1} KH_2PO_4, 0.25 g l^{-1} NaCl, 0.5 g l^{-1} NH_4Cl, 0.12 g l^{-1} $MgSO_4$, and 0.005 g l^{-1} $CaCl_2$, and 20 g l^{-1} agar, adjusted to pH 5.0) and the top layer contained 5 ml of a semi-opaque overlay medium (basal medium mixed with 10 g l^{-1} of colloidal chitin as chitinase substrate). A loop of yeast colony from section 2.5 was cross-streaked on the surface of the medium plate. All plates were incubated for 5 days at 28 ^{O}C in an incubator (Memmert, type UM 600, Germany) in the dark before observing for chitinase activity. A clear zone around the colony of yeast in a semi-opaque medium indicated the chitinase activity. The experiment was repeated three times.

4.7.4.2 Qualitative detection of β-1,3-glucanase activity in agar plate

Secretion of β-1,3-glucanase was investigated by streaking *P. guilliermondii* strain R13 from section 2.5 on YPD plate containing 0.2 % laminarin azure (laminarin covalently linked with Remazol Brilliant Blue) as the enzyme substrate. The plates were incubated for 5 days at 28 ^{O}C in the incubator in the dark and then β-1,3-glucanase activity was observed. A clear zone around the colony in the green background of the agar plate indicated β-1,3-glucanase activity. The experiments were performed in triplicate.

4.7.5 Quantitative detection of chitinase and β-1,3-glucanase activities from *P. guilliermondii* strain R13

4.7.5.1 Culture of yeast *P. guilliermondii* strain R13 in liquid media

P. guilliermondii strain R13 was cultured in a modified Vogel's (1956) medium by adding 0.1 g l^{-1} of yeast extract and supplementing with substrates, i.e., either glucose (0.5 g l^{-1}) or laminarin from *Laminaria digitata* (0.5 g l^{-1}), or colloidal chitin (0.5 g l^{-1}), or cell wall preparation (CWP, 0.5 g l^{-1}) from *C. capsici* as the sole carbon source.

For fungal cell wall preparation, *C. capsici* was cultured in 15 ml of malt extract broth containing malt extract (17 g l^{-1}), peptone (3 g l^{-1}) for 5 days at 28 ^{O}C in the incubator. The culture was centrifuged at 3000 × *g* to collect the mycelium which was washed several times with distilled water. Then the washed

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mycelium was homogenized for 2 min and centrifuged for 10 min at 3000 × *g* and the supernatant was discarded. The pellet was frozen at -70 ^{O}C and pulverized in a mortar with a pestle. The pulverized mycelium was re-suspended in an equal volume of 50 mM Tris/HCl buffer, pH 7.2, centrifuged for 10 min at 3000 × *g*, and the supernatant was discarded. The pellet was subjected to three successive cycles of centrifugation and re-suspension. The final pellet of cell wall preparation was stored at -20 ^{O}C until use.

Flasks (250 ml) containing 100 ml of culture media were incubated on a rotary shaker (200 rpm) at 28 ^{O}C for 120 h. Three separate flasks were used for each substrate. Cultures were centrifuged for 10 min at 3000 × *g* and the supernatant was filtered through a 0.22 μm filter (Millex-GP, Millipore, USA) and stored at -20 ^{O}C until used for enzyme essay.

4.7.5.2 Determination of β-1,3-glucanase activity

β-1,3-glucanase activity from yeast strain R13 was assayed according to the method of Bergmeyer and Brent (1974). 5 mg ml^{-1} of laminarin (from *Laminaria digitata*, Sigma L-9634) in 1 ml of 50 mM potassium acetate buffer, pH 5.0, was added to 1 ml of *P. guilliermondii* strain R13 culture filtrate (section 2.6.1). The reaction mixture was incubated at 37 ^{O}C in water bath for 30 min and terminated by keeping it in boiling water for 10 min. The enzyme activity was determined by following the amount of released glucose from laminarin by using God-Perid glucose test kit (Boehringer Mannheim, GMBH, Germany). The kit consists of glucose oxidase, peroxidase, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS). 3 ml of the glucose test kit reagent (1.5 mg ml^{-1}) were added to the reaction mixture, which were then incubated at 28 ^{O}C for 30 min before measuring for optical density at 420 nm. The amount of released glucose was determined using the glucose standard curve. Specific activity of β-1,3-glucanase was expressed as μmol glucose released/min/mg protein. Protein content of *P. guilliermondii* strain R13 was measured according to Bradford's method (1976) using bovine serum albumin (Sigma A-9647, Sigma Chemical Co.) as standard.

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4.7.5.3 Determination of chitinase activity

Chitinase activity was determined according to the method of Wirth and Wolf (1990), using colloidal chitin azure (chitin covalently linked with Remazol Brilliant Violet 5R dye) as substrate (Loewe Biochemica, Germany). 1 ml of 1 mg ml^{-1} colloidal chitin azure in 0.1 mM sodium acetate buffer, pH 5, was incubated with 1 ml of *P. guilliermondii* strain R13 culture filtrate (section 2.6.1) for 1 h at 37 ^{O}C. The reaction was stopped by adding 50 µl of 1 M HCl. The reaction mixture was cooled on ice for 10 min and centrifuged at 15000 × *g* for 5 min. The supernatants were measured for optical density at 550 nm (Beckman Coulter, DU© 800, USA). The unit of chitinase activity from *P. guilliermondii* strain R13 was determined from a standard curve of chitinase from *Streptomyces griseus* (Sigma C-6137).

4.7.6 Studies on production of killer toxin

P. guilliermondii strain R13 was tested for killer toxin production by using the well test technique (Somers and Bevan 1969). *Saccharomyces cerevisiae* strain NCYC 1006 (National Collection of Yeast Cultures, Norwich, UK) was used as the strain sensitive to the killer toxin (Farris *et al.*, 1991). *P. guilliermondii* strain R13 was preliminarily cultured in YEPD broth (1.0% yeast extract, 2.0% peptone, 0.1 M citrate phosphate buffer pH 5.0) at 28 ^{o}C for 5 days. The culture was filtered through a 0.45 µm millipore filters (Millipore Corp., Bedford, Mass.). 80 µl aliquot of yeast culture filtrate was added to a small well (8 mm in diameter) on YEPD-MB agar plate (YEPD containing 0.01% methylene blue and 2.0% agar) previously seeded with 0.1 ml of the 24-h culture of the sensitive strain (*S. cerevisiae* strain NCYC 1006). For a positive control of killer toxin production, *Kluveromyces lactis* killer strain CBS 2359 (Holland) was used (Aguiar and Lucas, 2000). Sterile distilled water was used as the negative control. All plates were incubated at 28 ^{O}C for 5 days, and then detected for killer toxin production. The growth inhibition zone of the sensitive strain around the well indicated production of the killer toxin. Each test was done in triplicate.

4.8 Statistical analysis

Data analysis of disease incidence, yeast biological control efficacy, lesion diameter, competition for nutrients**,** and hydrolytic enzyme activities were done by using the general linear model (GLM) procedure of SPSS software (version 10.0 for

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Windows, SPSS Inc., Chicago, Illinois, USA). The least significant difference (LSD) test at $P < 0.05$ was used for mean separation.

Educational part

Results from scientific investigation of antagonistic yeast for biological control of postharvest chilli anthracnose were applied for educational aspect. The hands-on yeast biological control experiment was developed for high school students for improving their concept and critical thinking. This designed experiment was incorporated into the topic of organism interrelationships in biology curriculum.

4.9 Contexts of the study

The hands-on yeast biological control experiment was developed as an interactive activity for improving students' concept and critical thinking through experiential learning model. The advantages of the experiential learning model are to help students assume responsibility for their own learning because it asks them to reflect on their experiences, draw conclusions, and identify applications. Learners ground the lesson in their actual environment by considering the question of what can or should be done differently as a result of the learning experience. Therefore, the experiential learning model was chosen in this study to enhance thinking skills, as well as to facilitate conceptual and attitudinal development. The study was conducted at the senior secondary school of Thailand. This school is designed to providing education for the full development of students in science and mathematics. This research works on the topic of organism interrelationship and performed on 2nd semester.

4.10 Research design

4.10.1 Population setting

Both qualitative and quantitative investigations were used in this study. The quasi-experiment was set to compare the two groups of senior secondary students in a science school in Thailand; the experiential learning and traditional teaching groups. The three week-studies on biological control were conducted in two classes of sixty-four students of the 11th grade enrolled in elective biology course. The 11th grade students were chosen to join this study because they already had necessary

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backgrounds of cell structure and function, diversity of organisms, and nutrition. They were randomly divided into two groups; the experiential learning and traditional learning group. The experiential learning group (16 male and 19 female) was taught according to the teaching sequence described in Table 1. The students had opportunities to do hand-on biological control experiment and designed their own projects. The traditional learning group (14 male and 17 female) was given only lecture without doing the hand-on experiment or designing science projects. The conceptual understandings of both groups were assessed by MCQ test, analysis of constructed concept map, and conceptual interviewing. Their critical thinking skills related to biological control context were evaluated by using critical questions

4.10.2 Teaching sequences

The teaching sequences were conducted according to the designed lesson plan based on experiential learning cycle of Kolb and Fly (1975). The process started with pretest of students' prior knowledge in both experiential learning and traditional lecture groups. The students in experiential learning group were then engaged to the lesson by the critical questions, followed by concrete experience with content discussion, hands-on experiment, and reading the selected biological control article. They practiced in observation and reflection that would lead to construction of the concepts or knowledge learned from the performed activities. At the end of the activities, they were asked to apply the constructed knowledge in a new situation, i.e., designing their own science projects. The reflections of conceptual understanding and critical thinking of students after the activities were evaluated by the constructed assessment tools. Student's attitudes toward the hands-on activity were also probed. Teaching sequences of experiential learning group was summarized in Figure 4.

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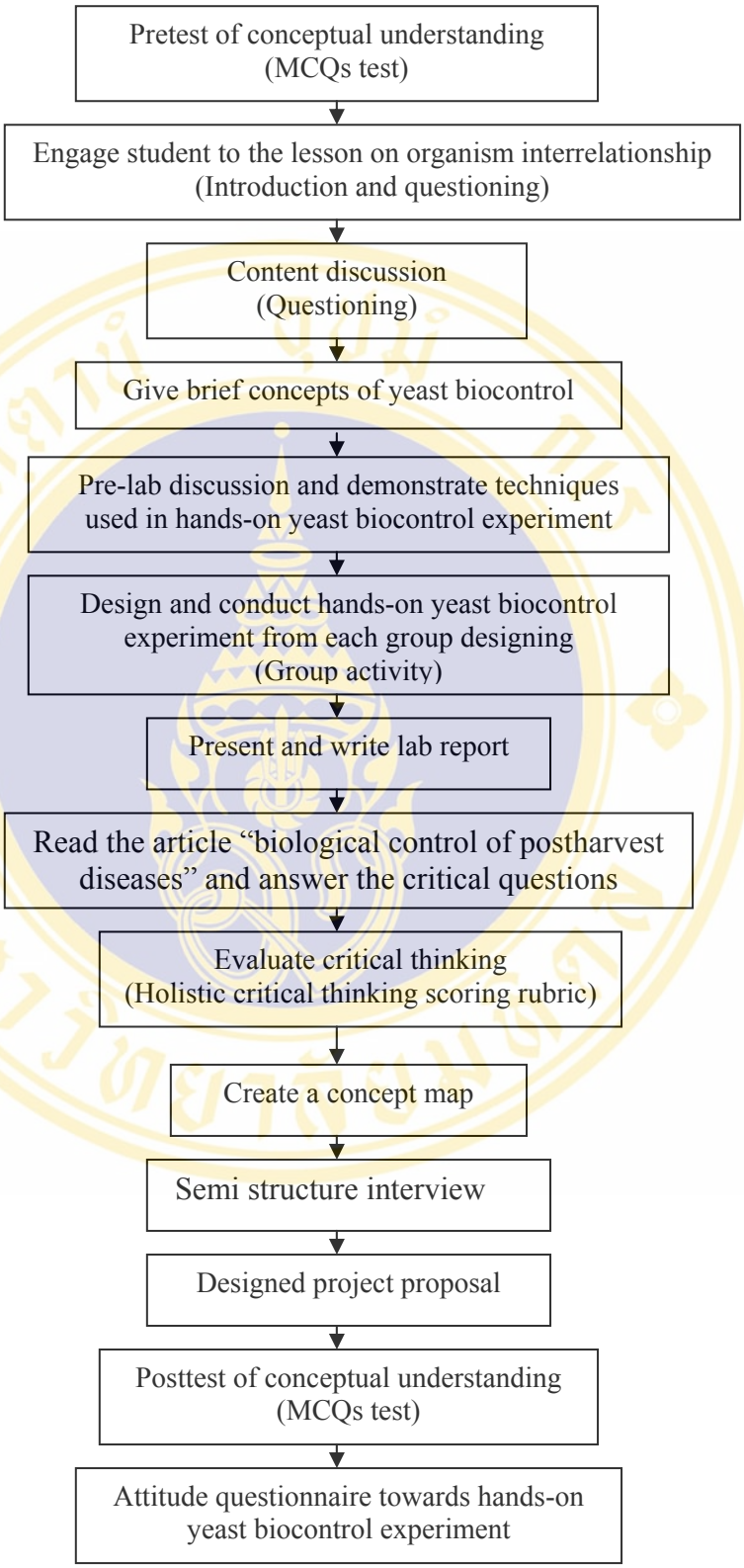

Figure 4-1 Teaching sequences of the experiential learning group.

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4.11 Development of lesson plan

The lesson plan was designed based on the experiential learning model

4.11.1 Lesson objectives

Students should investigate why individual organisms do not live in isolation in a community, how organisms interact with each other in the ecology system, how many types of interrelationship among them, and what the important roles of these relationships. At the end of activity, the students should be able to:

1) Define types of intra-specific interactions and inter-specific interactions in terms of the effects of organisms on each other

2) Distinguish each type of organism interrelationships

3) Analyze the roles of relationships to the balance of nature

4) Relate the knowledge of organism interrelationship to biological control and apply it to science project and diary life

5) Show the ability of critical thinking and scientific inquiry.

4.11.2 Lesson plan of experiential learning group

The lesson plan of experiential learning group was prepared before instruction. The class activities with the assessments of conceptual understanding and critical thinking skills would be completed in 3 weeks (100 min each week). The lesson plan and the time schedule are shown in Table 4-2.

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Table 4-2 Lesson plan of the experiential learning group.

Phase/Day	Activities	Time (min)
1	1) Pretest: students conduct pretest (multiple choices)	20
	2) Engagement: students are asked the following question: "Can individual organism live alone in the habitat?" And "Why did you answer that way?" The purpose of questioning is to engage students and tie them to the lesson.	10
	3) Briefing contents: teacher briefs the contents of organism interrelationships in the ecosystem and biological control.	20
	4) Demonstration: teacher demonstrates the hands-on yeast biological control of anthracnose disease caused by mold, *Colletotrichum capsici*, in chili fruit and basic microbiological techniques (aseptic techniques, media preparation, yeast and mold isolation from the surface of crops, yeast and mold culturing, and slide preparation for microscope observation).	25
	5) Experimental design: students work in groups of four and design the experiments to test their predictions. Their experimental design must include a hypothesis, control group, variables, and methodology. The teacher will approve student's plan prior to implementation.	25
2	6) Students conduct their own designed experiments. Teacher will act as facilitator. Students solve the problems from doing laboratories by brainstorming and sharing of ideas.	60
	7) Students' presentation: each group of student analyses the data and presents the results, including data tables, graphs comparing control and experimental groups, and conclusions to the class. Other groups should participate by sharing ideas in the discussion.	35
	8) Assignment: students are assigned lab write-up reports.	5 (and home work)
3	9) Reading an article: students are assigned to read the biological control article and answer the critical questions.	30
	10) Concept mapping: each group of students draws the concept map.	30
	11) Interview: teacher interviews individual student with concept questions	45 (after class)
	12) Project assignment: students are assigned to apply the knowledge gained to design proposal for the biological control project to be presented in group report.	10 (and after class)
	13) Posttest: students conduct posttest (multiple choices)	20
	14) Attitude survey: students answer the questionnaires in the attitude survey form	10

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4.12 Development of hands-on activity on yeast biological control experiment

The hands-on experiment on biological control was designed based on the research works on yeast biological control of anthracnose disease in chilli fruits (Chanchaichaovivat *et al.*, 2007). After content discussion of organism interrelationship in the ecosystem with special emphasis on biological control (about 20 min), the teacher demonstrated the methods used to control chilli anthracnose disease by antagonistic yeasts (25 min). The method involved isolation of yeast antagonists from fruits, how to make wounds on fruit samples, culturing methods of yeast antagonist and fungal pathogen in the laboratory, and inoculation of these microorganisms on plates and on fruit sample wounds. Groups of students then discussed to design their own experiments (25 min) e.g. choose types of fruit samples for isolation of yeast antagonists and for using as a host plant, choose fungal pathogen strain for biological control test (the teacher prepared broad range of fungal pathogens for the experiment, e.g., *Colletrotrichum* spp, *Botrytis* spp., *Rhizopus* spp.). The students set their own hypothesis of the experiment, and define the variables (such as concentration of yeast or fungal pathogen, type of host plant, temperature, incubation time, etc.). They should know how to collect and analyse. In the second day of activities, each group of students performed the experiment according to their own plans (60 min), recorded the data, and made laboratory report. Meanwhile, teacher acted as facilitator, the students discussed results and problems from laboratories through brainstorming and sharing the ideas.

4.13 Data collection

14.13.1 Pretest and posttest

The conceptual understanding of students prior to teaching in both traditional learning and experiential learning groups was evaluated by giving the pretest on conceptual questions (14 multiple choice questions; MCQs) for 20 min at the beginning of the teaching sequence. The MCQs test and the level of difficulty based on Bloom Taxonomy (1956) are shown in Appendix B. The same questions were used for posttest at the end of teaching sequence.

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4.13.2 Enhancing critical thinking skills

After finish the experiment, the students were assigned to read the article on “Biological Control of Postharvest Diseases” in class for 20 min and answered the questions shown in (Appendix C). These questions focused on the abilities to analyze, interpret, evaluate, inference, explain, and self-regulate which are the main components of critical thinking skills according to Facione and Facione (1994).

4.13.3 Concept mapping

Each group of students in the experiential learning group was asked to construct their concept maps to cover concepts on “organism interrelationship in biological control of plant diseases” and brainstorming. The teacher introduced concept mapping briefly to students individually for around five minutes and allowed students to ask questions while arranging their own concept maps. The concept map in each group was created within 30 min.

4.13.4 Conceptual Interviewing

The random sampling of eight students from experiential learning and traditional teaching classes were performed for semi-structured interviews after class on Day 3 (Table 4-2). Each 45 min-interview was audiotaped and researchers’ field notes were taken as an additional data source. The interview questions generated from the context of organism interrelationship in biological control of plant diseases are shown in Table 4-3.

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Table 4-3 Questions for semi-structured interview.

Questions	Concepts to be probed
1) What is the interrelationship between pathogen and plants? 2) What could be the interactions between 2 different types of microbe resided in the same habitat e.g. fruits or vegetables? 3) What could be interrelationships among human, plant pathogens, other microbes, fruits or vegetables, and insects?	Basic idea concerning the concepts of organism interrelationship
4) Why do microbes live with the plant? 5) How do the microbes compete with each other? 6) How does one organism inhibit the growth of other organism?	Basic concept regarding the modes of action between organisms
7) What is the meaning of biological control and its application? 8) What are the examples of biological control used in your country?	The concepts of biological control

4.13.5 Laboratory report

Individual student was assigned to write up the laboratory report after doing the yeast biological control laboratory. The students would need to write clearly and order their thoughts in a logical way throughout the report.

4.13.6 Designed project proposal

Each group of four students in the experiential learning group was asked to extend their knowledge to design a science project, in which they will perform for next two semesters. The students could ask for more guidance from teacher about the techniques in order to come out with the most plausible projects. To write a proposal, the students searched information from several resources including websites, organizations, journals, and experts. Students set their meeting schedules

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with the advisory teachers and reported the progress of their work plans once a week. After 1 month they presented and defended their proposals to the project committees, the project might be adapted and improved according to the comments.

4.14 Data analysis

The quasi-experimental model was adopted to compare two groups of senior secondary school students, experiential learning group and traditional learning groups. Statistical data analysis were accomplished by independent-samples t-test analysis ($P<0.05$) of SPSS software (version 10.0 for Windows, SPSS Inc., Chicago, Illinois, USA). The significance at $P < 0.05$ was used for mean separation. The assessment tools for evaluate each type of raw data were described in the following.

4.14.1 Pretest and posttest

Multiple choice questions (14 items) with four options were constructed to evaluate the conceptual understanding. The questions were based on the 6 levels according to Bloom's taxonomy in the cognitive domain (Bloom, 1956). Conceptual test was validated by IOC (Index of Consistency) method (Wainer and Braun, 1988). The comments from two experts in biology from the secondary school and an expert in the field of microbial ecology from the university were used to fine-tune the format and structure of the test. Then, the improved tools were tried out with 100 grade 11 Thai students who have previous knowledge on the organism interrelationship. Item analysis of the test consists of Cronbach alpha value (Cronbach, 1951), level of difficulty, discrimination power, intellectual level, and the overall reliability of the test are shown in Table 4-4.

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Table 4-4 The quality of multiple-choice test.

Item	Cronbach alpha value[a] (α)	Difficulty Index (pi)	Discrimination index (Di)
1	0.83	0.58	0.48
2	0.90	0.61	0.51
3	0.79	0.57	0.47
4	0.81	0.46	0.41
5	0.78	0.64	0.46
6	0.77	0.45	0.32
7	0.91	0.54	0.51
8	0.88	0.49	0.54
9	0.89	0.53	0.40
10	0.92	0.59	0.59
11	0.78	0.61	0.73
12	0.85	0.54	0.41
13	0.82	0.64	0.46
14	0.92	0.58	0.50

[a]The overall reliability of the test was α = 0.82.

To calculate the difficulty of each item, the number of persons answered correctly was divided by number of all answered persons. This proportion was designated by the letter *p,* which indicated the difficulty of the item (Crocker and Algina, 1986). It was calculated by the following formula:

$$p_i = \frac{A_i}{N_i}$$

where: *pi* = difficulty index of item *i*

Ai = number of correct answers to item *i*

Ni = number of correct answers plus number of incorrect answers to item *i*

According to the EXHCOBA manual: The Basic Knowledge and Skill Examination (Backhoff *et al.*, 2000), the difficulty level of the examination should range between 0.50 and 0.75, the values of *p* being distributed in the following manner: easy items, 5%; items of medium-low difficulty, 20%; items of medium difficulty, 50%; medium-hard items, 20%; and difficult items, 5%.

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To calculate the discrimination power (*Di*), the following formula was used:

$$D_i = \frac{GA_{\text{correct answers}} - GB_{\text{correct answers}}}{N_{\text{largest group}}}$$

where: *Di* = discrimination power of item *i*

$GA_{correct\ answers}$ = number of correct answers to item *I* among the 27% of those with highest test scores.

$GB_{correct\ answers}$ = number of correct answers to item *I* among the 27% of those with lowest test scores.

$N_{largest\ group}$ = number of person in the largest group (GA or GB)

Ebel and Frisbie (1986) have given the following rule of thumb for determining the quality of the items, in terms of the discrimination power (*Di*). Table 4-5 showed the values *Di* and their corresponding interpretation, and recommendations for each value.

Table 4-5 Discrimination power (*Di*) of the answers.

Di	Quality	Recommendations
> 0.39	Excellent	Retain
0.30-0.39	Good	Possibilities for improvement
0.20-0.29	Mediocre	Need to check/review
0.00-0.20	Poor	Discard or review in depth
<-0.01	Worst	Definitely discard

4.14.2 Critical thinking

The students' critical thinking skills were evaluated by using the Holistic Critical Thinking Scoring Rubric, HCTSR (Facione and Facione, 1994) consisted of 4 levels of critical thinking abilities: poor, fair, good, very good (Table 4-6). The critical thinking skills were also evaluated in the control group by using the same questions. Laboratory reports of the experimental group were evaluated for

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critical thinking abilities by using HCTSR. Additionally, the critical thinking of each student on the biological control method was evaluated by asking an open ended question: "Why do you prefer to use biological control method rather than chemical method for the planting?"

Table 4-6 Holistic critical thinking scoring rubric.

Critical thinking skills	Score
Consistently does all or almost all of the following: - Accurately interprets evidence, statements, graphics, questions, etc. - Identifies the salient arguments (reasons and claims) pro and con. - Thoughtfully analyzes and evaluates major alternative points of view. - Draws warranted judicious, non-fallacious conclusions. - Justifies key results and procedures, explains assumptions and reasons. - Fair-mindedly follows where evidence and reasons lead.	4 (very good)
Does most or many of the following: - Accurately interprets evidence, statements, graphics, questions, etc. - Identifies relevant arguments (reasons and claims) pro and con. - Offers analyses and evaluations of obvious alternative points of view. - Draws warranted, non-fallacious conclusions. - Justifies some results or procedures, explains reasons. - Fair-mindedly follows where evidence and reasons lead.	3 (good)
Does most or many of the following: - Misinterprets evidence, statements, graphics, questions, etc. - Fails to identify strong, relevant counter-arguments. - Ignores or superficially evaluates obvious alternative points of view. - Draws unwarranted or fallacious conclusions. - Justifies few results or procedures, seldom explains reasons. - Regardless of the evidence or reasons, maintains or defends views based on self-interest or preconceptions.	2 (fair)
Consistently does all or almost all of the following: - Offers biased interpretations of evidence, statements, graphics, questions, information, or the points of view of others. - Fails to identify or hastily dismisses strong, relevant counter arguments. - Ignores or superficially evaluates obvious alternative points of view. - Argues using fallacious or irrelevant reasons, and unwarranted claims. - Does not justify results or procedures, nor explain reasons. - Regardless of the evidence or reasons, maintains or defends views based on self-interest or preconceptions. - Exhibits close-mindedness or hostility to reason.	1 (poor)

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4.14.3 Concept mapping

Students' concept maps in organism interrelationship were evaluated by holistic concept map scoring rubric adapted from Moni *et al.* (2004) as shown in Table 4-7. They were marked in three probing criteria, i.e., content, logic and understanding, and presentation. Maximum score of each criterion is 5 and overall core is 15. At the maximum quality: all relevant concepts of organism interrelationship are correct with multiple connections; understanding of facts and concepts of organism interrelationship is clearly demonstrated by correct links; and concept map is neat, clear, and legible, has easy-to-follow links and has no spelling errors. On the other hand, in the minimum total score of 3, the concepts are linked via the linking words but it is consisted of low interrelationship between concepts and hard to understand.

Table 4-7 Holistic concept mapping scoring rubric.

Score	Content	Logic and Understanding	Presentation
5	All relevant concepts of organism interrelationship are correct with multiple connections.	Understanding of facts and concepts of organism interrelationship is clearly demonstrated by correct links.	Concept map is neat, clear, and legible, has easy-to-follow links and has no spelling errors.
4	Most relevant concepts of organism interrelationship are correct with multiple connections.	Understanding of facts and concepts of organism interrelationship is demonstrated by a few error links.	Concept map is neat, clear, and legible, has easy-to-follow links and has some spelling errors.
3	Few relevant concepts of organism interrelationship are correct with two or more connections.	Understanding of facts and concepts of organism interrelationship is demonstrated but some incorrect links.	Concept map is neat, legible but with some links difficult to follow and has some spelling error.
2	Few relevant concepts of organism interrelationship are correct with no connections.	Understanding of facts and concepts of organism interrelationship with some significant errors.	Concept map is untidy with links difficult to follow and has some spelling error.
1	The concepts are linked via the linking words		

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4.14.4 Laboratory report

Students' laboratory reports were evaluated by scoring rubric adapted from Merced Country Office of Education (2006) as shown in Table 4-8.

Table 4-8 Laboratory report scoring rubric.

Criteria	Beginning (1 point)	Developing (2 points)	Accomplished (3 points)	Exemplary (4 points)
Introduction	Does not give any information about what to expect in the report	Gives very little information	Gives too much information-more like a summary	Presents a concise lead-in to the report
Hypothesis	Hypothesis has major flaws and limited or no relevancy	Hypothesis is partially developed with some relevancy	Hypothesis has been sufficiently developed with reasonable relevancy	Hypothesis has been thoroughly developed with correct stated variables identified
Purpose	Demonstrates no understanding of purpose	Demonstrates limited understanding of purpose	Demonstrates significant understanding of purpose	Demonstrates total understanding of purpose
Procedure	Not sequential, most steps are missing or are confusing	Some of the steps are understandable; most are confusing and lack detail	Most of the steps are understandable; some lack detail or are confusing	Present easy-to follow steps which are logical and adequately detailed
Data and results	Data table and/or graph missing information and are inaccurate	Both complete, minor inaccuracies and/or illegible characters	Both accurate, some ill-formed characters	Data table and graph neatly completed and totally accurate
Discussion and Conclusion	Presents an illogical explanation for findings and does not address any of the questions	Presents an illogical explanation for findings and addresses few questions	Presents a logical explanation for findings and addresses some of the questions	Presents a logical explanation for findings and addresses most of the questions
Attractiveness	Illegible writing, missing pages	Legible writing, some ill-formed letters, print too small or too large	Legible writing, well-formed characters, clean and neatly bound, illustrations provided	Word processed, clean and neatly bound, illustrations provided

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4.14.5 Interviewing

The conceptual interviews were transcribed from answering of students according to the questions in section 4.13.4 and then evaluated by scoring rubric consisted of the criteria, i.e., basic idea concerning the concepts of organism interrelationship, basic concept regarding the modes of action between organisms, and the concepts of biological control (Table 4-9). Each criterion was evaluated according to 4-level scoring rubric: very good (all answers are clear and accurate), good (all answers are rather clear and understanding), fair (some or all answers are ambiguous or misconception in the relationship between the given concepts), and poor (the student can not answer all questions). The data were analysed for the misconception of each criterion.

Table 4-9 Interview scoring rubric.

Probing concepts	1 point	2 points	3 points	4 points
1. Basic idea concerning the concepts of organism interrelationship	The student can not answer all questions.	Some or all answers are ambiguous or misconception in the relationship between the given organisms.	All answers are rather clear and understanding.	All answers are clear and accurate.
2. Basic concept regarding the modes of action between organisms	The student can not answer all questions.	Some or all answers are ambiguous or student can not explain how the organisms interact with each other.	All answers are rather clear and understanding.	All answers are clear and accurate.
3. The concepts of biological control	The student can not answer all questions.	Some or all are ambiguous or misconception in biological control method.	All answers are rather clear and understanding.	All answers are clear and accurate.

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4.14.6 Project proposal

The project proposals were evaluated by scoring rubric adapted from Handerson (1997) consisting of abilities to identify the objectives, hypothesis, variable and control, procedure, and data interpretation (Table 4-10). Each attribute was evaluated in scores at three levels: 10-8 points = above standard, 7-5 points = at standard, 4-0 points = below standard.

Table 4-10 Project proposal rubric.

Attributes	Superior task (10-8 points)	Adequate task (7-5 points)	Inadequate task (4-0 points)	Attribute points earned
Purpose	Identified a question (without the teacher's assistance) which they found interesting and testable; utilized literature search to develop a hypothesis which was reasonable.	Identified a question (with the teacher's assistance) which they found interesting and testable.	The purpose is incomplete, too easy to attain, or does not follow from your research.	—
Variable and control within the purpose	Variables which are to be changed (independent) and variables that are going to be measured are clearly defined. The group is committed to analyzing an ambitious number of variables that will result in a thorough study of the defined purpose.	Variables which are to be changed (independent) and variables that are going to be measured are clearly defined. The group has committed to analyzing a number of variables that will result in a thorough study of the defined purpose.	Variables which are to be changed (independent) and variables that are going to be measured are not clearly defined. The group's defined variables will result in an incomplete study of the defined purpose.	—

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Table 4-10 Project proposal rubric (continued).

Attributes	Superior task (10-8 points)	Adequate task (7-5 points)	Inadequate task (4-0 points)	Attribute points earned
Hypothesis	Utilized literature search to develop a hypothesis which was reasonable and well substantiated with research.	Utilized literature search to develop a hypothesis which was reasonable.	Hypothesis is not complete or does not flow logically from research.	—
Procedure	A well thought out, sequential (step-by-step) procedure is stated that anyone could look at and follow. It holds high promise for collecting the information sought. Measurements to be made are systematic and logically controlled (changing one variable at a time) and are repeated to improve reliability of data.	A well thought out, sequential (step-by-step) procedure is stated that anyone could look at and follow. It holds high promise for collecting the information sought. The measurements to be made are systematic and logically controlled (changing one variable at a time).	The procedure is incomplete, not sequential, or takes effort on the part of the reader to follow. It may not be systematic or logically controlled (perhaps your group has defined many variables to vary at once and have not clearly decided how to measure all variables.)	—
Diagram (s)	The diagram(s) are present that lay out the nature of your experiment. The computer generated diagram(s) clearly show (with labels)	The diagram(s) are present that lay out the nature of your experiment. The diagram(s) clearly show (with labels)	The diagram(s) are incomplete, hard to follow or missing.	—

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Table 4-10 Project proposal rubric (continued).

Attributes	Superior task (10-8 points)	Adequate task (7-5 points)	Inadequate task (4-0 points)	Attribute points earned
Data interpretation plan	Plans for displaying the collected data are clearly laid out (a table is strongly recommended). Thoughts for ambitious analysis of data (graphical analysis, etc.) are clearly communicated.	Plans for displaying the collected data are clearly laid out (a table is strongly recommended). Thoughts for thorough analysis of data (graphical analysis, etc.) are clearly communicated.	The plan is incomplete or does not logically match with the data your group has decided to collect.	—
Total proposal points earned				—

4.14.7 Students' attitudes towards yeast hands-on experiment

The students were investigated for their attitudes towards learning hands-on yeast biological control experiment by using a questionnaire with five point Likert's scale (5 = strongly agree, 4 = agree, 3 = neutral, 2 = disagree, 1 = strongly disagree). The questionnaires consist of statements about the benefits from participating in yeast biological control hands-on in the as part of conceptual understanding and thinking skills (Table 4-11).

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Table 4-11 Students' attitudes survey form.

Item	Scale[a]				
	1	2	3	4	5
1) This hands-on experiment provides better understanding in the concept of organism interrelationship.					
2) I have the opportunities to practice microbiological techniques.					
3) This hands-on experiment encourages me to think scientifically.					
4) This hands-on experiment helps me know better about microorganism.					
5) This hands-on experiment is interesting.					
6) I can apply this hands-on experiment to design the science project.					
8) This hands-on experiment inspires me to think about the effects of synthetic fungicides to the environment.					
9) This hands-on experiment can be applied in my daily life.					
Other commentaries:					

[a]The scales: 1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, 5 = strongly agree

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CHAPTER V
RESULTS

This chapter presents the results of yeast antagonist screening from fruits and vegetables. These yeast strains showed the various abilities to control postharvest chilli anthracnose caused by *Colletotrichum capsici*. The modes of action of the best effective yeast antagonist were also studied in the competition for nutrients, hydrolytic enzymes and killer toxin productions. These results were support the high biological control efficacy of the yeast antagonist. Moreover, the results from using the knowledge of yeast biological control for *C. capsici* showed the effectiveness of hands-on experiment of yeast biological control to enhance conceptual understanding and critical thinking of 11th grade students through the experiential learning model.

Scientific part

The results in scientific part are regarding the isolation and screening of yeast antagonists from surfaces of fruits and vegetables. These yeast strains showed the different abilities to control anthracnose disease caused by *C. capsici* both *in vitro* and *in vivo* tests. The effectiveness of postharvest disease control of the antagonistic yeasts compared with chlorinated water was presented. The modes of action of the best effective yeast antagonist to *C. capsici* were also revealed in this part.

5.1 Yeast isolation from fruits and vegetables

As a result of the isolation procedure, 225 yeast isolates were obtained from 7 species of fruits and 5 species of vegetables (section 4.1.2). The highest number of yeast isolates were obtained from rambutan (38 isolates, 16.9%), whereas the lowest yeast number (7 isolates, 3.1%) were found from bird pepper (Table 5-1). Isolated yeast colonies were observed in white, cream, orange-brown, and pink-red at 72.22%, 16.67%, 7.41% and 3.70%, respectively. All of 225 isolates were next screened for antagonism to anthracnose pathogen, *C. capsici*.

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Table 5-1 Percentage of yeast isolates from various species of fruits and vegetables.

Source	Scientific name	Yeast Code	Number of yeast isolate (%)
Rambutan	*Nephelium lappaceum* L.	R1 - R38	16.9
Pineapple	*Ananus comosus* Merr.	P1 – P29	12.9
Longan	*Dimocarpus longan* Lour.	L1 - L25	11.1
Banana	*Musa sapientum* L.	B1 - B21	9.3
Sweetbell pepper	*Capsicum annuum* L. var. *grossum*	SP1 - SP19	8.4
Rose apple	*Eugenia javanica* Lamk.	RA1 - RA18	8.0
Mango	*Mangifera indica* L.	M1 – M17	7.6
Chilli	*Capsicum annuum* L. var. *acuminatum* Fingerh.	C1 - C15	6.7
Sapodilla	*Achras sapota* L.	S1 – S14	6.2
Plate brush	*Solanum torvum* Swartz	PLB1 – PLB12	5.3
Eggplant	*Solanum melongena* L.	ER1 – ER10	4.4
Bird pepper	*Capsicum frutescens* L.	BP1 – BP7	3.1
Total		225 isolates	

5.2 Screening for yeast antagonists against *C. capsici*

The primary screening of yeast antagonists from 225 yeast isolates was tested by their abilities to resist fungal invasion on plate culture technique (Figure 5-1). Yeast isolates were streaked on PDA plates that seeded with spore of *C. capsici*. The results showed that some yeast isolates had ability to inhibit fugal growth, which could be observed the dark brown mycelium of *C. capsici* against the yeast colonies and the fungus could not proliferate to the yeast cell areas (Figure 5-1a). By this technique, it was resulted in 54 positive yeast isolates. All of them were further tested in the secondary screening method.

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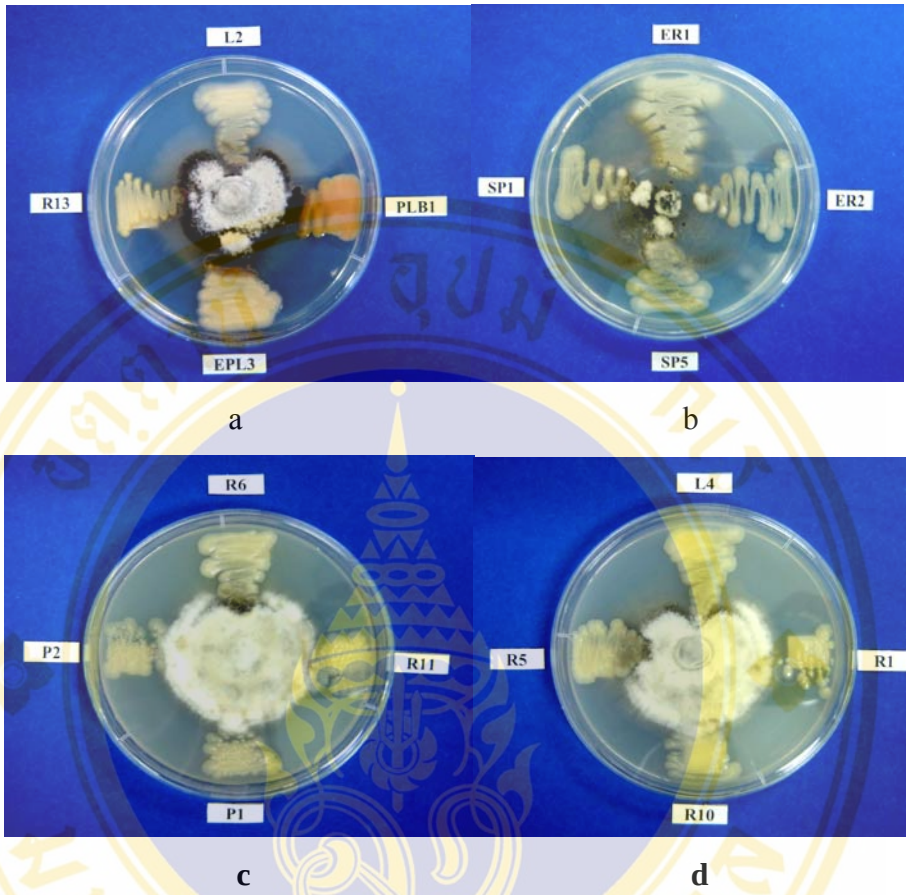

Figure 5-1 Primary screening of antagonistic yeasts. The 225 yeast isolates were streaked on PDA plates seeded with *C. capsici* ($5x10^4$ spore ml^{-1}) and incubated at 28 ^{O}C for 5 days. The samples of yeast isolates showed different abilities to resist *C. capsici* invasion on PDA plates. (a) Yeast isolate R13, L2, (b) yeast isolate ER1, SP1, SP5, ER2, (c) yeast isolate R6, and (d) yeast isolate L4 were selected as antagonistic yeasts.

Secondary screening of these 54 isolates for biological control efficacy against *C. capsici* growth using the well test (section 4.2.3) showed that only four yeast strains were effective in inhibition of *C. capsici* growth, i.e., no mycelial growth on the PDA plates as observed under the microscope (Figure 5-2). In these four yeast strains, two were isolated from rambutan, these are assigned R13 and R6 and other two strains from red eggplant and longan, ER1 and L2, respectively. These four yeast strains were tested further for confirmation of their potential in controlling chilli anthracnose caused by *C. capsici*.

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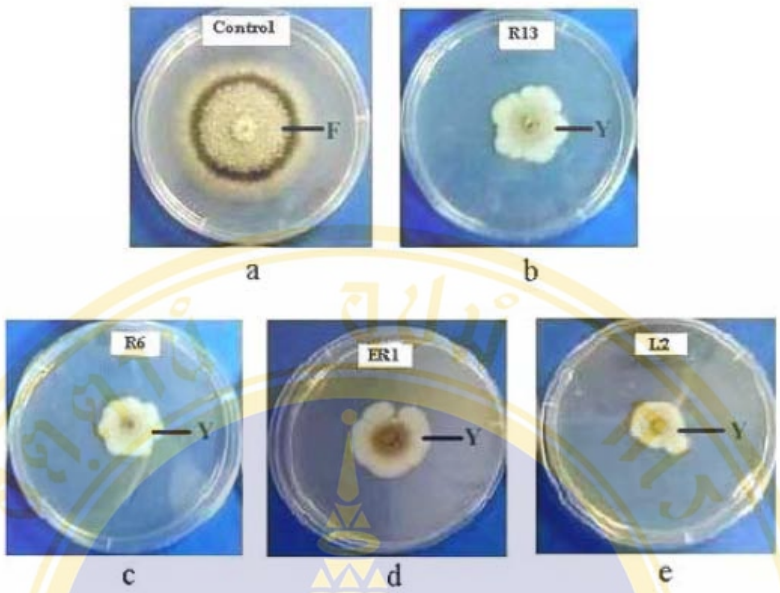

Figure 5-2 Biocotrol assay by using *in vitro* test of yeast isolates (R13, R6, ER1 and L2) showing colonny of *C. capsici* in control group without yeast inoculation (a) and inhibition of *C. capsici* growth by yeast strain R13 (b), R6 (c), ER1 (d) and L2 (e) on PDA plates. (F, fugal mycelia; Y, yeast cell mass).

5.3 Identification of the yeast antagonists

5.3.1 Identification of yeast antagonists by rDNA sequence comparison

Yeast strains R13, R6, ER1, and L2 with the ability to control *C. capsici* growth in the *in vitro* test were analysed for 26S (D1/D2 region). The sequence analysis of large subunit (26S) ribosomal DNA gene of yeast strains R6, ER1, and L2 showed high identity with those of *C. musae*, *I. orientalis*, and *C. quercitrusa*, i.e., 97%, 99%, and 99%, respectively (Table 5-2). However, identification of R13 by analysis of large subunit 26S rDNA showed 99% identity with *P. guilliermondii*, *C. fukuyamaensis*, and *C. xestobii*. Thus the small subunit of 5.8S rDNA (TSI1/TSI4 region) was further used for more effective identification (Kurtzman, 1992; Martorell *et al.*, 2005). The results showed 98% identity of R13 to *P. guilliermondii* (Table 5-2).

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Table 5-2 Identification of the four strains of yeast isolates by the rDNA sequencing and morphological characteristics (colony, cell, and spore morphologies).

Yeast isolate	Identity[a] (%)	rDNA[b] region	Species
R13	98	ITS1/ITS4	*Pichia guilliermondii*
R13	99	D1/D2	*Pichia guilliermondii*
			Candida fukuyamaensis
			Candida xestobii
R6	97	D1/D2	*Candida musae*
ER1	99	D1/D2	*Issatchenkia orientalis*
L2	99	D1/D2	*Candida quercitrusa*

[a]The percentage identity among DNA fragments was calculated with BLAST program and the sequences were compared with those from NCBI database.

[b]Region of the rDNA gene used for identification.

5.3.2 Morphological and physiological characteristics of yeast antagonists

Yeast strains R13, R6, ER1, and L2 were found to be different in colony characteristics and cell morphologies as shown in Table 5-3 and Figure 5-3. They are all oval shape with different size, which yeast strain ER1 shows the biggest size (Figure 5-3) and strain R13 are in the smallest size. These two stains can produce ascospore in the ascus, whereas, it did not found in strain R6 and L2 (Table 5-3). The results of physiological characteristics of yeast strains according to fermentation and assimilation tests (Table 5-4) agreed with the data of the rDNA analysis (Table 5-2). The different patterns of carbon fermentation and assimilation of R13, R6, ER1, and L2 as shown in Table 5-4 indicate that R13 has abilities to ferment and assimilate more carbon sources than others, while ER1 showed abilities to ferment and assimilate very few carbon sources in this system. All of the 4 yeast antagonists cannot utilise lactose, however, they can utilize glucose, D-glucosamine, and glycerol as sole carbon source (Table 5-4).

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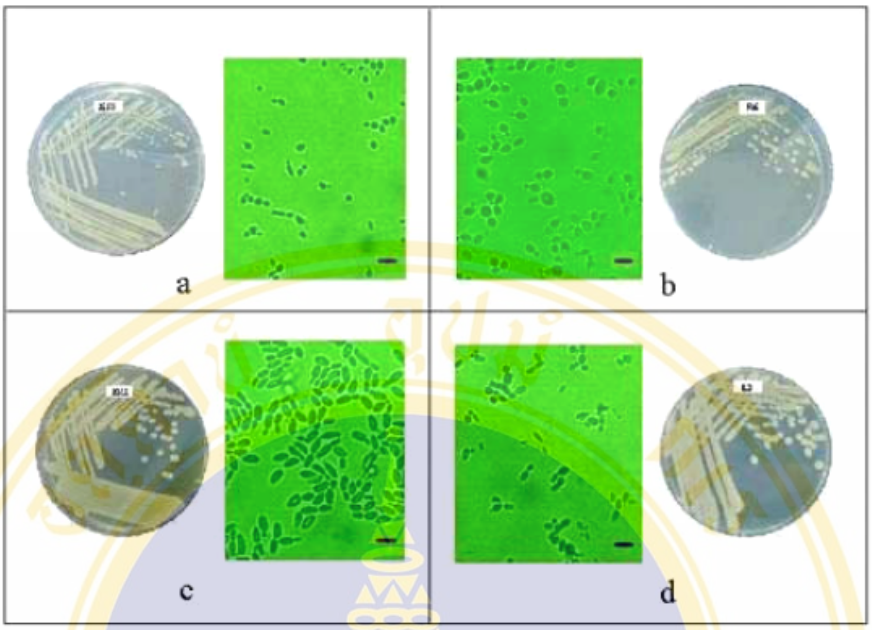

Figure 5-3 Colony characteristics and cell morphologies of yeast strain R13 (a), R6 (b), ER1 (c), and L2 (d) cultured on 5 % malt extract agar (strain R13 and ER1) and corn meal agar (strain R6 and L2) cell morphologies were observed under a microscope (bar = 10 µm).

Table 5-3 Morphological characteristics (colony, cell, and spore morphologies) of the four strains[a].

Yeast isolate	Colony morphology	Cell morphology	Spore morphology
R13	tannish-white, butyrous	ovoidal (1.5-4.8) x (2.0-15.0) µm, singles, pairs, short chains	hat-shaped, 1-4 spores per ascus
R6	white, creamy	ovoidal (3.0-5.0) x (4.0-8.0) µm, singles, small groups	no spore
ER1	light cream, butyrous	ovoidal to elongate, (1.3-6.0) x (3.3-14.0) µm, singles, pairs	spherical, 1 spore per ascus
L2	white, butyrous	ovoidal, (2.0-5.0) x (4.5-7.0) µm, pseudohyphae	no spore

[a]Yeast strains were grown on a medium recommended by Kurtzman and Fell (1998).

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Table 5-4 Comparison of fermentation and assimilation tests (using biochemical reactions) of the four effective yeast strains (R13, R6, ER1, and L2) with *Pichia guilliermondii*, *Candida musae*, *Issatchenkia orientalis*, and *Candida quercitrusa* as listed by Kurtzman and Fell (1998).

Test	Strain R13	*P. guilliermondii*	Strain R6	*C. musae*	Strain ER1	*I. orientalis*	Strain L2	*C. quercitrusa*
Fermentation:								
D-Glucose	+	+	+	+	+	+	+	+
D-Galactose	-	v	-	-	-	-	-	v
Sucrose	+	+	-	-	-	-	-	-/s
Maltose	-	-	-	-	-	-	-	-/s
Lactose	-	-	-	-	-	-	-	-
Raffinose	+	+	-	-	-	-	-	-
Trehalose	+	+	+	s	-	-	-	-
Assimilation:								
D-Glucose	+	+	+	+	+	+	+	+
D-Galactose	+	+	-	-	-	-	+	+
L-Sorbose	+	v	+	+	-	-	+	+
D-Glucosamine	+	+	+	l	+	+	+	+
Actidione	+	NL	-	NL	-	NL	-	NL
Saccharose	+	+	+	+	-	-	+	+
N-acetyl-D-glucosamine	+	+	+	l	+	+	+	+
DL-Lactate	-	v	-	-	+	+	+	l
L-Arabinose	+	+	-	-	-	-	-	-
Cellobiose	+	+	-	-	-	-	-	-
Raffinose	+	+	-	-	-	-	-	-
Maltose	+	+	+	+	-	-	+	+
Trehalose	+	+	+	+	-	-	+	+
2-Keto-D-gluconate	+	+	+	+	-	-	+	+
α-Methyl-D-glucoside	+	+	-	l	-	-	+	+
D-Glucitol	+	+	+	+	-	-	+	l
D-Xylose	+	+	+	+	-	-	+	v
D-Ribose	+	+	-	-/l	-	-	+	l
Glycerol	+	+	+	+	+	+	+	+
L-Rhamnose	-	v	-	-	-	-	-	-
Palatinose	+	NL	+	NL	-	NL	+	NL
Erythritol	-	-	-	-	-	-	-	-
Melibiose	+	+	-	-	-	-	-	-
D-Glucuronate	-	NL	-	-	-	NL	-	-
Melezitose	+	+	+	+	-	-	+	+
D-Gluconate	+	v	+	l	-	-	+	+
Levulinate	-	NL	-	NL	-	NL	-	NL
D-Mannitol	+	+	+	+	-	-	+	l
Lactose	-	-	-	-	-	-	-	-
myo-Inositol	-	-	-	-	-	-	-	-

+, positive reaction; -, negative reaction; v, variable reaction; s, positive but slow reaction; -/s, negative or positive but slow reaction; l, latent reaction (rapidly developing a positive reaction after a lag phase); -/l, negative or latent reaction; NL, test results not listed by Kurtzman and Fell (1998).

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5.4 Studies on severity of *C. capsici*

A preliminary experiment showed that *C. capsici* at concentrations from $5x10^2$ to $5x10^4$ spores ml^{-1} caused lesion diameters on chilli fruits from 6.7 to 15.8 mm. The spore concentrations of more than $5x10^4$ spores ml^{-1} did not produce significant increase in lesion diameter (Figure 5-4). Thus the fungal concentration of $5x10^4$ spores ml^{-1} was used in the subsequent experiments.

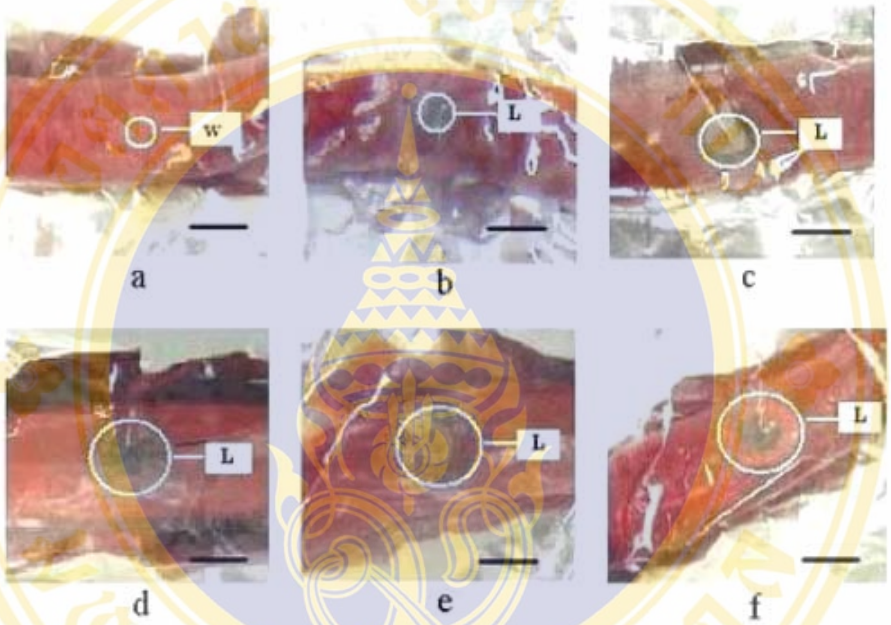

Figure 5-4 Effects of various *C. capsici* concentrations on lesion diameter in chilli fruits (a) no spore, (b) $5x10^2$, (c) $5x10^3$, (d) $5x10^4$, (e) $5x10^5$ and (f) $5x10^6$ spores/ml. (W, wound; L, lesion area; bar = 1 cm).

5.5 Comparison of *in vivo* biological control efficacy of the four antagonistic yeast strains

The biological control efficacy of the four antagonistic yeast strains was confirmed by using the *in vivo* test, or by their demonstrated ability to reduce disease incidence in chilli fruits. The four strains of yeast isolates with high biological control efficacies, selected based on data from the *in vitro* tests (section 5.2) were tested to inhibit *C. capsici* growth on chilli wound. Figure 5-5 shows the lesion diameters of wounded chilli fruits that reduced by the 4 yeast antagonists (strains R13, R6, ER1, and L2) compared with the control (Figure 5-5b) inoculated with only *C. capsici*

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spores. Yeast stain R13 inhibits anthracnose appearance quite better than the rest three stains after 5 days of chilli storage (Figure 5-5c). The abilities to reduce lesion diameter of chilli are yeast strain R13, strain R6, strain ER1, and L2 respectively. The results in Table 5-5 show that strain R13 had the highest biological control efficacy of 93.3%, or disease incidence of only 6.7%. Yeast strains R6, ER1, and L2 showed biological control efficacies of 83.1%, 76.6%, and 66.4%, respectively. Moreover, the four yeast antagonists were significantly effective ($P < 0.05$) in reducing the diameter of lesion caused by *C. capsici*, i.e., from 15.4 mm in *C. capsici* infection to 6.7, 7.8, 9.1, and 10.3 mm upon treatment with R13, R6, ER1, and L2 respectively (Table 5-5).

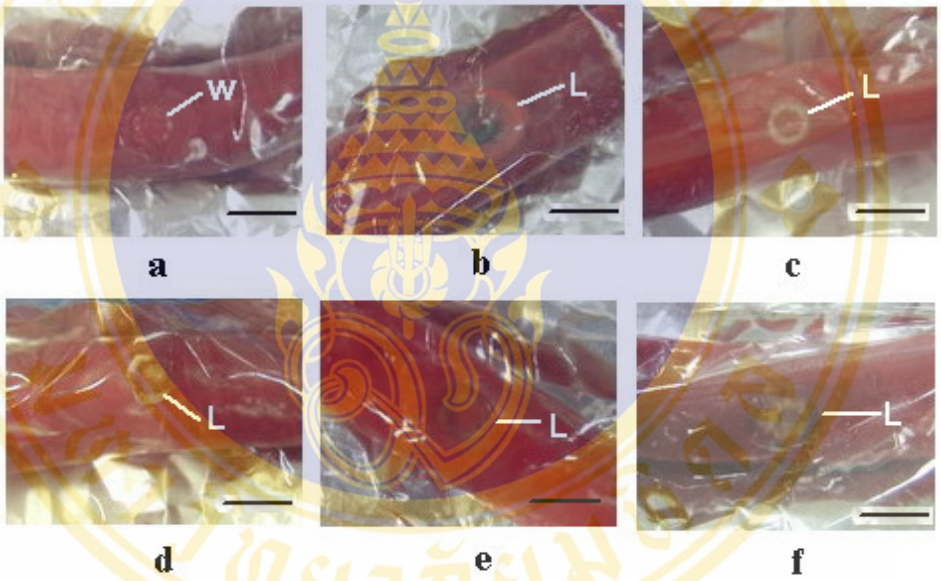

Figure 5-5 Biological control efficacies of four yeast antagonists against *C. capsici* on chilli wounds. Each yeast strain at concentration of $5x10^9$ cells ml^{-1} was applied to the wound of chilli wound. Then 20 µl of *C. capsici* at a concentration of $5x10^4$spores ml^{-1} was added to the wound. Chilli fruits were stored in the dark at 28 ^{O}C. Lesion diameter in chilli fruits was detected 5 days after storage. (a) Chilli wound inoculated with only distilled water, (b) Inoculation with only *C. capsici*, (c) *C. capsici* and yeast isolate R13, (d) *C. capsici* and yeast isolate R6, (e) *C. capsici* and yeast isolate ER1, and (f) *C. capsici* and yeast isolate R6. (W, wound; L, lesion area; bar = 1 cm).

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Table 5-5 The effectiveness of the four antagonistic yeast strains in reduction of disease (anthracnose) incidence in *Colletotrichum capsici* infected chilli fruits.

Yeast isolate	Disease incidence[a] (%)	Biological control efficacy[a] (%)	Lesion diameter[b] (mm)
Pichia guilliermondii R13	6.7 ± 0.40a	93.3 ± 0.40a	6.7 ± 0.23a
Candida musae R6	16.9 ± 0.87b	83.1 ± 0.87b	7.8 ± 0.17b
Issatchenkia orientalis ER1	23.4 ± 0.92c	76.6 ± 0.92c	9.1 ± 0.28c
Candida quercitrusa L2	33.6 ± 0.52d	66.4 ± 0.52d	10.3 ± 0.35d
Control	100.0 ± 0.00e	0.0 ± 0.00e	15.4 ± 0.40e

[a]%Disease incidence = (A/T) x 100 and %Biological control efficacy = [(T - A)/T] x 100, where T is the number of infected wounds of chilli fruits inoculated with *Colletotrichum capsici* only (control), and A is the number of infected wounds of chilli fruits inoculated with both yeast antagonists and *C. capsici*. The results are presented as mean of three independent experiments ± standard error. Values of each column followed by a different letter indicate significant difference ($P < 0.05$) according to LSD test.

[b]Lesion diameter is the average length of lesion in x-axis and y-axis.

5.6 Effects of antagonistic yeast concentration on disease control

The results of testing different concentrations of the four yeast strains against *C. capsici* at various concentrations show that the yeast strain R13 is more effective than the other three strains (R6, ER1, and L2) in reducing disease incidence at all concentrations tested (Figure 5-6). Upon infection with $5x10^6$ spores ml^{-1} of *C. capsici*, the disease incidence was reduced from 23.3% to 10.5% as concentrations of R13 cells increased from $5x10^6$ to $5x10^8$ cells ml^{-1}. However, R6, ER1, and L2 strains reduced the disease incidence to lesser extent, i.e., from 25.8% to 15.6%, 28.2% to 17.9%, and 33.5% to 22.5% respectively. Control group with only *C. capsici* ($5x10^4$ - $5x10^6$ spores ml^{-1}) infection showed 100% disease incidence (data not shown).

At lower *C. capsici* concentrations of $5x10^5$ and $5x10^4$ spores ml^{-1}, disease incidence decreased from 20% to 6.7% and 13.3% to 6.5% as R13 increased from $5x10^6$ to $5x10^8$ cells ml^{-1} (Figure 5-6a). However, increasing concentrations of the

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yeast strain R13 from $5x10^8$ to $5x10^9$ cells ml^{-1} did not result in greater reduction in disease incidence caused by $5x10^6$-$5x10^9$ spores ml^{-1} of *C. capsici*. Similar findings were observed in the yeast strains R6, ER1, and L2 although the extent of disease reduction was smaller (Figure 5-6 b, 5-6c, and 5-6d).

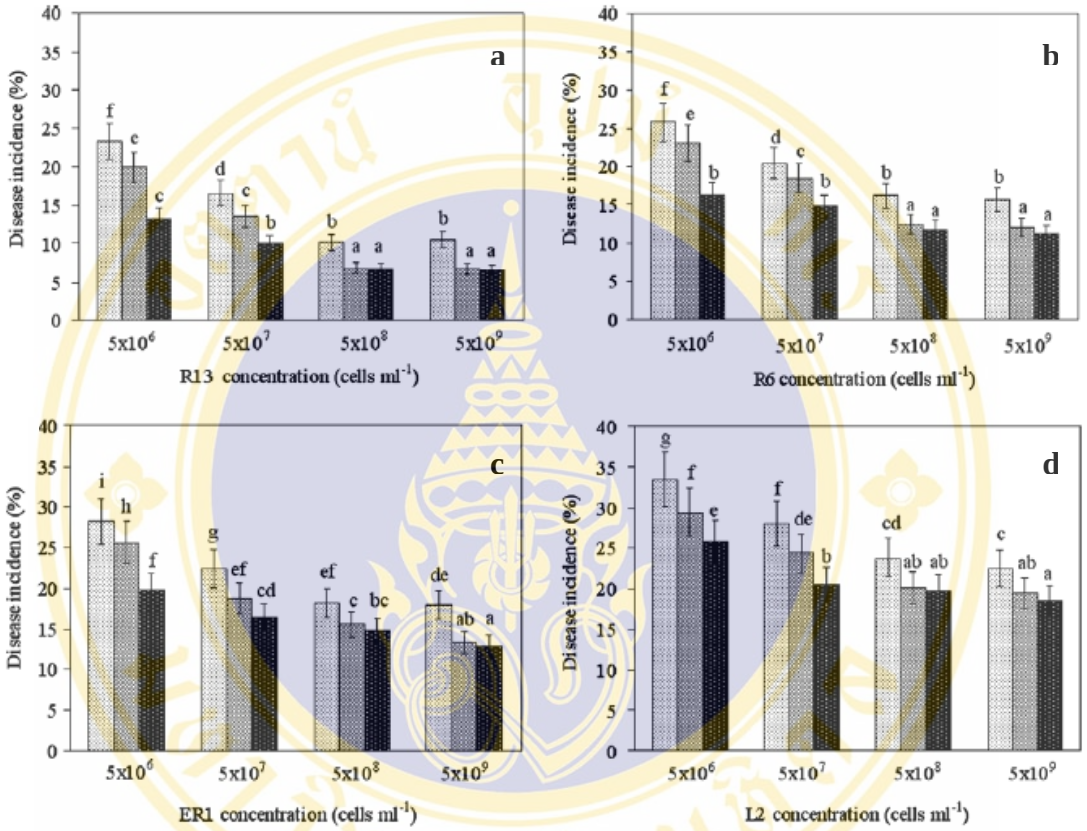

Figure 5-6 Effects of yeast concentration on reduction of disease (anthracnose) incidence in chilli fruit. Thirty wounded fruits were inoculated with 20 µl of the yeast antagonists: *Pichia guilliermondii* R13 (a), *Candida musae* R6 (b), *Issatchenkia orientalis* ER1 (c), and *Candida quercitrusa* L2 (d) at concentrations of $5x10^6$, $5x10^7$, $5x10^8$, and $5x10^9$ cells ml^{-1}. After air drying, the fruits were inoculated with 20 µl of different concentrations of *Colletotrichum capsici*: $5x10^6$ spores ml^{-1} (▒), $5x10^5$ spores ml^{-1} (▒), and $5x10^4$ spores ml^{-1} (▒). Data were recorded five days after storage at 28 ^{O}C. Bars represent the means of three independent experiments ± standard errors. Letters on the top of each bar indicate the results of LSD test ($P < 0.05$). Values with shared letters in the same graph are not significantly different. Control with only *C. capsici* infection resulted in 100% disease incidence.

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5.7 Effects of temperature on disease control by yeast antagonists

Yeast strain R13 was significantly superior to the other three yeast strains in reduction of disease incidence at all temperatures (18-33 ^{O}C) tested over a period of five days (Figure 5-7). The antagonistic effects of the four yeast strains were inversely related to the storage temperature. The biological control was more efficacious at 18 ^{O}C compared to that at 23, 28, and 33 ^{O}C. The disease incidences in R13 treatment were 1.1%, 3.3 %, 6.7%, and 10.0% at 18, 23, 28, and 33 ^{O}C respectively. At 33 ^{O}C, the disease incidence in R13 treatment was about 2, 4, and 5 times lower than that of R6, ER1 and L2 treatments. Control with only *C. capsici* inoculation showed disease incidence of 100% at storage temperature of 18-33 ^{O}C.

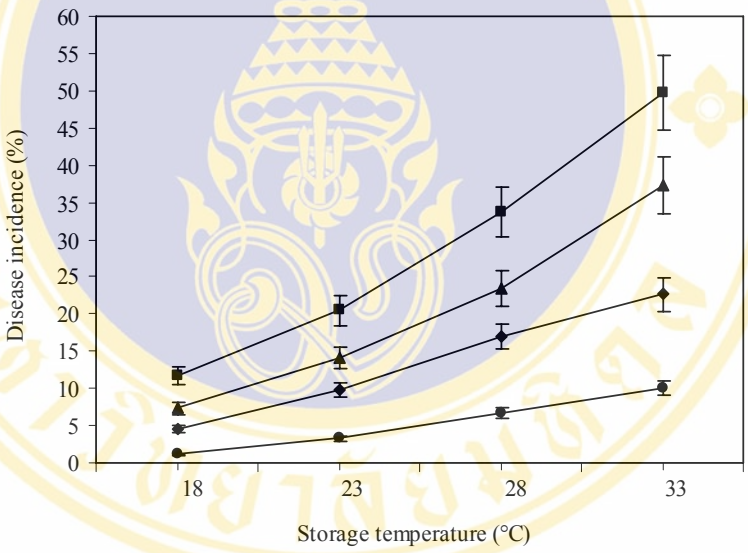

Figure 5-7 Effects of incubation temperature on percentage of disease (anthracnose) incidence on chilli fruits. Thirty wounded fruits were inoculated with 20 µl of yeast antagonists: *Pichia guilliermondii* R13 (●), *Candida musae* R6 (♦), *Issatchenkia orientalis* ER1 (▲), and *Candida quercitrusa* L2 (■) at concentration of 5x10^{8} cells ml^{-1}. After air drying, the fruits were inoculated with 20 µl of *Colletotrichum capsici* at concentration of 5x10^{4} spores ml^{-1}, and then incubated at 18, 23, 28, and 33 ^{O}C. Data were recorded five days after incubation. Bars represent standard errors of the mean of triplicate experiments. Control with only *C. capsici* infection resulted in 100% disease incidence at all temperatures tested.

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5.8 Postharvest disease control by yeast antagonists

Disease incidence in chilli fruits sprayed with R13 cells was significantly less than those treated with the other three yeast strains at all storage times (15, 30, 45 days) tested (Table 5-6 and Figure 5-8). Although the disease incidence gradually increased with storage time in all treatments, the R13 treatment was more efficacious than that of chlorinated water at 30 and 45 days of storage. The percentage of disease incidence in chilli fruits sprayed with R13 cell suspension and chlorinated water were 13.3% and 16.7%, 16.7% and 20.0% at 30 and 45 days of storage respectively (Table 5-6). In all cases, the disease incidences after yeast and chemical treatments were significantly less than that washed with only distilled water.

Table 5-6 Percentage of disease (anthracnose) incidence in intact chilli fruits sprayed with the four antagonistic yeasts, chlorinated water, and distilled water.

Treatment	Disease incidence[a] (%)		
	15 days	30 days	45 days
Pichia guilliermondii R13	3.3 ± 0.69a	13.3 ± 0.29a	16.7 ± 0.48a
Candida musae R6	4.4 ± 0.29ab	14.4 ± 0.40a	18.1 ± 0.35b
Issatchenkia orientalis ER1	5.1 ± 0.23b	17.5 ± 0.26b	21.4 ± 0.66d
Candida quercitrusa L2	5.7 ± 0.52bc	19.4 ± 0.46c	23.7 ± 0.29e
Chlorinated water	3.3 ± 0.46a	16.7 ± 0.42b	20.0 ± 0.46c
Distilled water	6.7 ± 0.41c	23.3 ± 0.40d	26.7 ± 0.34f

[a]The results are presented as mean of three independent experiments ± standard error. Values of each column followed by a different letter indicate significant differences ($P < 0.05$) according to LSD test. Values with shared letter are not significantly different ($P < 0.05$).

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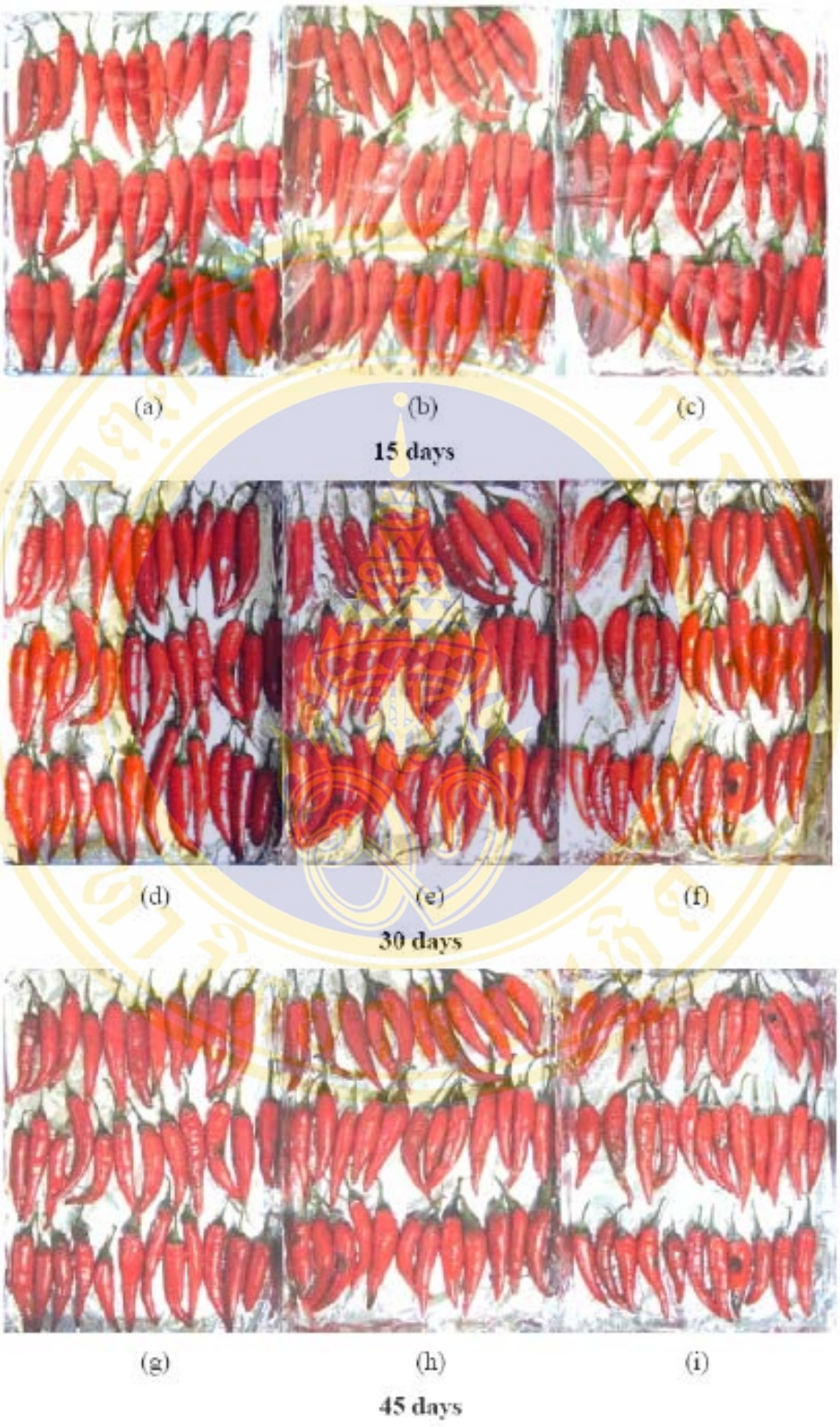

Figure 5-8 Postharvest biological control of chili fruits by spraying with *P. guiliermondii* R13. (a, d, g) chlorinated water (b, e, h) and distilled water (c, f, i). They were stored at 10 ^{o}C for 15, 30, and 45 days.

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5.9 Modes of action of yeast antagonist, strain R13 on *C. capsici* infected chilli fruits

The antagonistic yeast *P. guilliermondii* strain R13 isolated from rambutan has been shown to have highest biological control efficacy for chilli anthracnose caused by *C. capsici* The modes of action of this yeast antagonist against *C. capsici* were included attachment ability, competition for nutrients, secretion of hydrolytic enzymes, and killer toxin production.

5.9.1 Effects inoculation of yeast *P. guilliermondii* strain R13 and *C. capsici* into chilli fruits' wounds

Figure 5-9 showed a photograph of chilli anthracnose caused by *C. capsici*. Light microscopic examinations of thin sections of the chilli wounds showed a massive colonization of *C. capsici.* Proliferation of the pathogen in wounded tissue resulted in the collapse of cell walls and internal disorganization (Figures 5-10a and 5-10b). As shown in Figures 5-10c and 5-10d, invading hyphae and acervuli that embedded into the parenchyma tissue beneath the wound site caused extensive cellular degradation. Cell walls of the invaded host cells and those adjacent to them were severely damaged and darkened spots appeared on chilli tissues. *C. capsici* cells also grew inter- and intracellular of the cell layer immediately beneath the wound site. However, fungal colonization of the chilli wounds was observed in chilli tissue treated with *P. guilliermondii* strain R13 and then inoculated with *C. capsici* (Figure 5-10e). Examination of 30 thin sections from thirty different fruits all showed a decrease in numbers of invading hyphae in the treated tissues. Few fungal hyphae were detected in the wounded tissue layer treated with *P. guilliermondii*, and the invading hyphae were usually surrounded by the antagonistic yeast cells. *P. guilliermondii* multiplied in the wound site and formed a matrix of yeast cells that covered plant-cell layers ruptured during wounding (Figures 5-10e and 5-10f) resulting in preservation of the parenchyma tissue beneath the wound site (Figure 5-10f). Wounded tissue treated only with *P. guilliermondii* showed no sign of cellular alterations (Figure 5-10g). The healthy chilli tissues without yeast and fungal pathogen inoculation are shown in Figure 5-10h.

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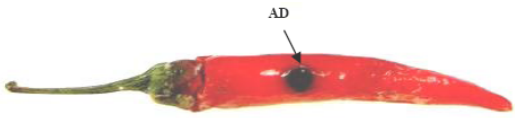

Figure 5-9 Anthracnose disease (AD) on a chili fruit at 5 days after inoculation with *C. capsici* into the wound.

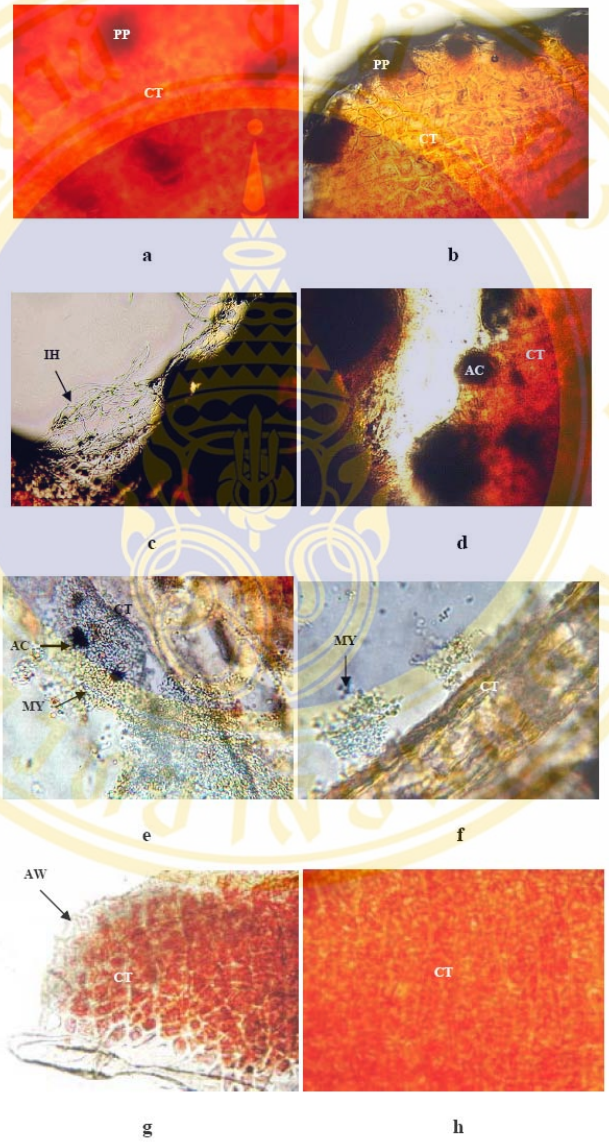

Figure 5-10 a) A massive colonization of *C. capsici* in the chilli wounds appeared as the dark spots. b) Pathogens proliferated (PP) in wounded tissue resulting in the collapse of chilli tissue, CT. c) Acervuli (AC) and d) Invading hyphae (IH) that embedded into the parenchyma tissue beneath the wound site causing extensive cellular degradation. e) and f) *P. guilliermondii* strain R13 multiplied in the wound site and formed a matrix of yeast cells (MY) covering plant-cell layers. g) The area of the wound (AW) preserved by *P. guilliermondii* cells. h) Healthy chilli tissues without yeast and pathogen inoculation.

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5.9.2 Studies on the attachment of *P. guilliermondii* strain R13 to hypha of *C. capsici*

Light microscopic observation of attachment of *P. guilliermondii* strain R13 to hypha of *C. capsici* done by applying cellophane disk to the inoculated chilli wounds indicated complete colonization of the yeast cells on the cellophane disks. At 12 h of incubation, the spore of *C. capsici* was surrounded by the yeast cells (Figure 5-11a) and the hyphal tip of germinating *C. capsici* spores were often clumped by yeast cells upon prolonged incubation to 20 h (Figure 5-11b). When the *C. capsici* was allowed to grow for 36 h before adding *P. guilliermondii*, the yeast cells attached tightly to *C. capsici* hyphae (Figure 5-11c, 5-11d and 5-11e). In the absence of *P. guilliermondii*, all the spores of *C. capsici* germinated and formed dense mycelia having hyphae with streaming cytoplasm (data not shown).

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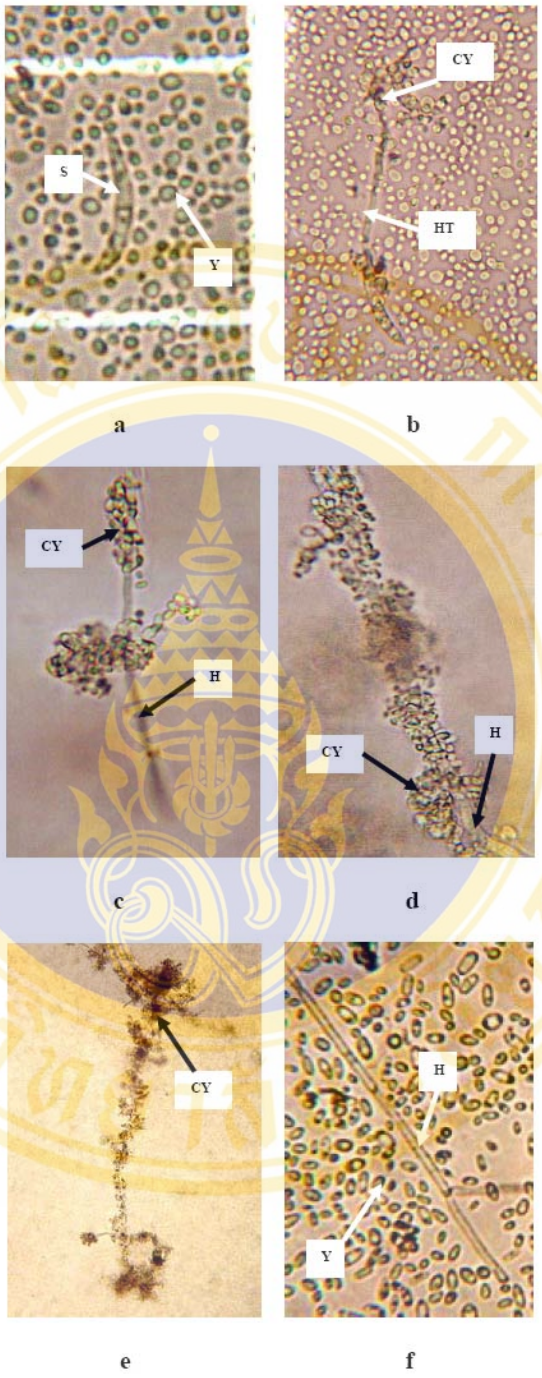

Figure 5-11 Attachment of *P. guilliermondii* strain R13 to spore and hypha of *C. capsici*. The interaction between yeast and fungus was studied on cellophane on the surface of chili wound. a) At 12 h inoculation, *C. capsici* spores (S) were surrounded by yeast cells (x 1,700). b) At 20 h of incubation, clump of yeast cells (CY) encircled the hyphal tip (HT) of spore germinated tube of *C. capsici* (x 1,000). c) and d) *P. guilliermondii* cells (Y) attached to *C. capsici* hypha (H) (x 1,000). e) Lower magnification (x 100) of *C. capsic* hypha clumped by *P. guilliermondii* cells. f) Lack of attachment of yeast cells (*Issatchenkia orientalis* strain ER1) on *C. capsic* hypha (x 1,000).

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5.9.3 Competition for nutrient by *P. guilliermondii* strain R13

The potential role of competition for nutrients in the biological control of *C. capsici* by strain R13 was examined by investigating the effects of increasing concentrations of three sugars and six nitrates on spore germination and germ tube length. Increasing the concentrations of glucose and sucrose from 0.05 to 2.00% nullified the suppression of spore germination and germ tube length (Table 5-7), suggesting that the yeast competed with *C. capsici* for these sugars. However, increasing concentrations of fructose did not increase germination or germ tube length (Table 5-7). As a control for the competition for nutrients, 0.05 to 2.00% glucose, sucrose, or fructose was added to cultures of either *C. capsici* or strain R13; the sugars did not inhibit spore germination of either organism, but increased germ tube length (Table 5-7). Addition of supplemental sugars to juice innocualted with only yeast strain R13 enhanced growth of the yeast by 25% (data not shown).

All nitrates tested except $Fe(NO_3)_3 \cdot 9H_2O$ nullified the inhibition by the antagonistic yeast although to a different extent (Table 5-8). $NaNO_3$ appeared to be the most effective in alleviating antagonism by strain R13. The failure of $Fe(NO_3)_3 \cdot 9H_2O$ to alleviate the antagonism is likely due to a fungistatic effect of $Fe(NO_3)_3 \cdot 9H_2O$; in the control, where nitrates were added to *C. capsici* culture in the absence of strain R13, $Fe(NO_3)_3 \cdot 9H_2O$ significantly inhibited *C. capsici* spore germination. However, other nitrate sources at the concentration of 0.1% did not inhibit spore germination of *C. capsici* or growth of strain R13 (Table 5-8). Addition of $Fe(NO_3)_3 \cdot 9H_2O$ to yeast strain R13 alone in chilli juice resulted in suppression of yeast growth, while other nitrates enhanced the growth by 10% (data not shown).

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Table 5-7 Effects of increasing concentrations of glucose, sucrose, and fructose on inhibition of spore germination and germ tube elongation of *C. capsici* by the antagonistic yeast *P. guilliermondii* strain R13.

Treatment	Sugar concentration (%)	Spore germination[a] (%)	Germ tube length[a] (μm)
juice + *C. capsici*	-	97.1 ± 5.37e	249.5 ± 10.05e
juice + *C. capsici* + yeast	-	7.3 ± 1.44a	75.9 ± 3.87a
Juice + *C. capsici* + glucose	0.05	98.3 ± 4.27e	387.6 ± 9.64g
	0.10	97.9 ± 3.4 7e	467.2 ± 11.54h
	0.20	97.6 ± 5.12e	570.9 ± 9.34i
Juice + *C. capsici* + yeast + glucose	0.05	22.3 ± 2.94b	110.4 ± 7.28b
	0.10	43.7 ± 5.37c	164.7 ± 8.72c
	0.20	62.5 ± 3.70d	195.6 ± 10.28d
Juice + *C. capsici* + fructose	0.05	96.8 ± 2.64e	257.9 ± 7.23e
	0.10	97.4 ± 4.87e	287.2 ± 9.78f
	0.20	98.0 ± 3.78e	291.7 ± 11.42 f
juice + *C. capsici* + yeast + fructose	0.05	26.7 ± 2.60b	123.3 ± 7.39b
	0.10	28.1 ± 3.35b	125.7 ± 8.20b
	0.20	24.9 ± 2.35b	119.2 ± 6.29b
juice + *C. capsici* + sucrose	0.05	97.4 ± 6.01e	298.4 ± 9.45f
	0.10	98.1 ± 5.27e	373.1 ± 8.97g
	0.20	98.7 ± 5.97e	449.6 ± 9.89h
juice + *C. capsici* + yeast + sucrose	0.05	15.4 ± 2.14b	97.3 ± 3.93b
	0.10	29.5 ± 2.43c	128.4 ± 7.74c
	0.20	47.3 ± 4.10d	172.5 ± 9.64d

[a]Spore germination and germ tube length were measured by microscopic micrometer after incubation for 16 h at 28 ^{O}C. The results are presented as means of three independent experiments ± standard error. Values of each column followed by a different letter indicate significant difference ($P < 0.05$) according to LSD test. Details of the experiment and control were as described in Methods section 4.7.3.

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Table 5-8 Effects of various nitrate sources on biological control efficacy of the antagonistic yeast *P. guilliermondii* strain R13 on spore germination and germ tube elongation of *C. capsici*.

Treatment	Spore germination[a] (%)	Germ tube length[a] (μm)
Juice + *C. capsici*	97.1 ± 5.37f	249.5 ± 10.05e
Juice + *C. capsici* + yeast	7.3 ± 1.44a	75.9 ± 3.87a
Juice + *C. capsici* + $Fe(NO_3)_3 \cdot 9H_2O$	6.7 ±2.58a	71.5 ± 6.25a
Juice + *C. capsici* + yeast + $Fe(NO_3)_3 \cdot 9H_2O$	5.8 ± 1.39a	68.3 ± 3.58a
Juice + *C. capsici* + $Ca(NO_3)_2 \cdot 4H_2O$	96.8 ±2.01f	260.9 ± 9.78ef
Juice + *C. capsici* + yeast + $Ca(NO_3)_2 \cdot 4H_2O$	35.7 ± 2.48b	120.5 ± 8.83b
Juice + *C. capsici* + $Mg(NO_3)_2 \cdot 6H_2O$	96.9 ±1.97f	267.3 ± 11.02ef
Juice + *C. capsici* + yeast + $Mg(NO_3)_2 \cdot 6H_2O$	44.2 ± 3.00bc	141.9 ± 9.87bc
Juice + *C. capsici* + KNO_3	97.5 ±1.84f	280.1 ± 11.67f
Juice + *C. capsici* + yeast + KNO_3	53.4 ± 3.64c	164.2 ± 11.26c
Juice + *C. capsici* + NH_4NO_3	98.4 ±2.94f	289.6 ± 11.97f
Juice + *C. capsici* + yeast + NH_4NO_3	68.6 ± 4.5d	189.5 ± 5.83d
Juice + *C. capsici* + $NaNO_3$	97.7 ±3.47f	301.5 ± 9.14g
Juice + *C. capsici* + yeast + $NaNO_3$	74.2 ± 5.25e	209.1 ± 6.70d

[a]Spore germination and germ tube length were measured by microscopic micrometer after incubation in the presence or absence of 0.1% of various nitrate sources for 16 h at 28 ^{O}C. Means ± standard error of each column followed by the different letters are of significant difference ($P < 0.05$) according to LSD test. Values followed by shared letter are not significantly different ($P < 0.05$). Details of the experiment and control were as described in Methods section 4.7.3.

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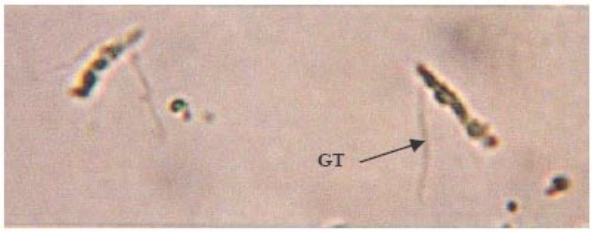

Figure 5-12 Germination tubes of *C. capsici* conidia (GT) in chilli juice after incubation for 16 h.

5.9.4 Production of hydrolytic enzymes by *P. guilliermondii* strain R13 in plate cultures

Preliminary test for chitinase activity was performed by using conventional plate method in which colloidal chitin was incoporated into an agar-medium base optimal for growth of *P. guilliermondii* strain R13. The chitinase enzyme cleaves the β-1,4-glycosidic bonds of chitin into oligomers and monomers resulting in a clear zone around the yeast colony. Figure 5-13 showed a clear zone of chitinase activity of *P. guilliermondii* strain R13 at 5 days after incubation; degradation of chitin in the medium caused clear zone around the yeast colony at the approximate diameter of 2.46 cm (Figure 5-13a).

a **b**

Figure 5-13 a) Clear zone of chitinase activity around the colony of *P. guilliermondii* strain R13 on chitin medium after incubation for 5 days. b) Opaque medium of colloidal chitin without yeast inoculation.

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P. guilliermondii strain R13 cultured on YPD plate containing 0.2% laminarin azure as a substrate showed a clear zone around the yeast colony at the diameter of 3.32 cm in 5 days of incubation (Figure 5-14a). The results suggested that the yeast produced β-1,3-glucanase (laminarinase) which then hydrolyzed the β-1,3-linkage of the laminarin azure causing disappearance of the dye colour.

a **b**

Figure 5-14 a) Clear zone of β-1,3-glucanase activity around the colony of *P. guilliermondii* strain R13 in laminarin azure medium after incubation for 5 days. b) Laminarin azure medium without yeast inoculation.

5.9.5 Secretion of hydrolytic enzymes by *P. guilliermondii* strain R13 in liquid media

The filtrate from 120-h culture of *P. guilliermondii* strain R13 in Vogel's (1956) medium was assayed for β-1,3-glucanase and chitinase activities. Fig. 5-15 showed β-1,3-glucanase specific activity of *P. guilliermondii* strain R13 cultured in three different carbon sources. Cell wall preparation induced the highest amount of β-1,3-glucanase specific activity at 7.58 μmol glucose released/30 min/mg protein, followed by laminarin and glucose at 3.69 and 0.42 μmol glucose released/30 min/mg protein, respectively (Figure 5-15). The results showed that cell wall of *C. capsici* induced β-1,3-glucanase activity at 2.1 times and 18 times higher than laminarin and glucose.

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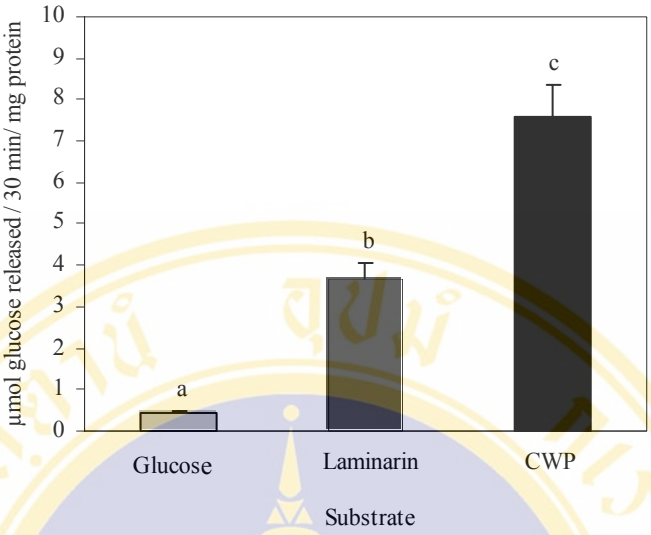

Figure 5-15 β-1,3-glucanase activity in the culture filtrate of *P. guilliermondii* strain R13 grown for 120 h at 28 ^{O}C in Vogel's medium amended with three different carbon sources [glucose (▒), laminarin (▓), and cell wall preparation (CWP) (■)]. β-1,3-glucanase activity was measured at 37 ^{O}C for 30 min using laminarin as substrate. Bars represent the means of three independent experiments ± standard errors. The different letters on the top of each bar indicate significant differences ($P < 0.05$) according to LSD test.

Similar results were obtained for expression of chitinase specific activity (µU chitinase /µg protein per hour) when *P. guilliermondii* strain R13 was cultured with the three carbon sources, i.e., glucose, colloidal chitin, and CWP. As shown in Figure 5-16, CWP induced the highest amount of chitinase specific activity at 32.84 µU chitinase /µg protein, followed by colloidal chitin and glucose at 24.89, and 2.75 µU chitinase /µg protein, respectively. The results showed that cell wall of *C. capsici* induced chitinase specific activity at 1.3 times and 11.9 times higher than colloidal chitin and glucose.

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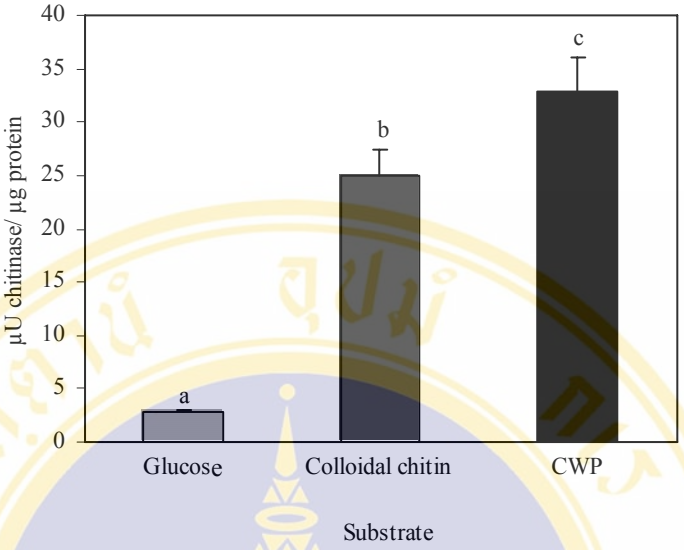

Figure 5-16 Chitinase activity in the culture filtrate of *P. guilliermondii* strain R13 grown for 120 h at 28 ^{O}C in Vogel's medium amended with three different carbon sources [glucose (▒), colloidal chitin (▓), and cell wall preparation (CWP) (■)]. Chitinase activity was measured at 37 ^{O}C for 1 h by using chitin azure as substrate. One unit will liberate 1.0 mg of N-acetyl-D-glucosamine from chitin per hour at pH 5.0 at 37 ^{O}C. Each assay was performed in triplicate and the entire experiment was repeated three times. Bars represent the means of three independent experiments ± standard errors. The different letters on the top of each bar indicate significant differences ($P < 0.05$) according to LSD test.

5.9.6 Production of killer toxin

Figure 5-17 showed the well test for killer toxin production from *P. guilliermondii* strain R13. The yeast *Saccharomyces cerevisiae* NCYC 1006 was used as the killer-sensitive strain. *Kluveromyces lactis* CBS 2359, the killer toxin production strain was used as positive control. After 5 days of incubation, *P. guilliermondii* strain R13 did not inhibit the growth of *S. cerevisiae* NCYC 1006 (Figure 5-17a), while the *K. lactis* CBS 2359 showed inhibitory effect on growth of *S. cerevisiae* NCYC 1006, as shown in the clear zone around the well (Figure 5-17c). The results suggested no killer toxin production by *P. guilliermondii* strain R13.

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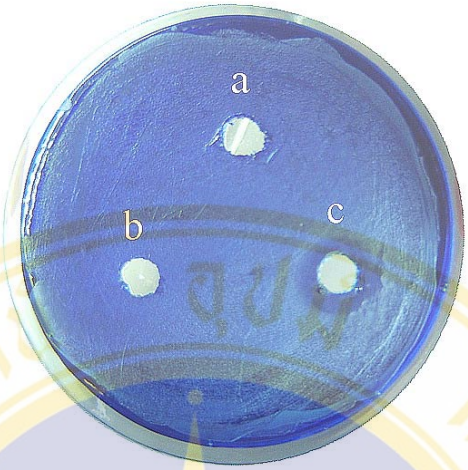

Figure 5-17 Well test of killer toxin production. YEPD-MB plate seeded with the sensitive yeast strain of *Saccharomyces cerevisiae* NCYC 1006 was incubated at 28 ^{O}C for 5 days with (a) culture filtrate of *P. guilliermondii* strain R13, (b) sterile distilled water, and (c) culture filtrate of *Kluveromyces lactis* CBS 2359 (clear zone around the well).

5.9 Enhancing conceptual understanding and critical thinking in biological control by using experiential learning

The results of research work on yeast biological control of anthracnose disease in chilli were used to develop the hands-on activities for the secondary students. The findings in this section showed how the success of this research in applying experiential learning on biological control experiment into 11th grade class for enhance conceptual understanding and critical thinking skills of students in biology course.

5.9.1 Conceptual understanding of students on biological control and organism interrelationship

The results in Table 5-9 show that students in the traditional and experiential learning groups have similar prior knowledge on biological control and organism interrelationship as illustrated by no significant difference in the pretest scores between the two groups. However, at the end of the teaching sequences, the

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posttest scores in the experiential learning group was significantly higher (P<0.05) than those in the traditional group.

Table 5-9 Pretest and posttest scores on conceptual understanding in the experiential learning and traditional groups.

	Experiential learning group		Traditional group	
	Pretest	Posttest	Pretest	Posttest
Mean score (max.14 points)[a]	4.27a	8.73b	4.58a	6.68c
Standard deviation	1.38	1.73	1.63	1.68
Number of students	33	33	31	31

[a]Mean values follow by different labeled letters are significantly different according to t-test analysis (P<0.05).

5.9.2 Students' critical thinking skills

In critical thinking assessment, Table 5-10 showed the mean scores of student's critical thinking skills from answers to the given questions after reading the assigned article on logical control of plant diseases. Mean comparison by independence t-test analysis revealed that students in the experiential learning group had significantly higher critical thinking skills than those in the traditional group in all questions tested (P<0.05). The students in experiential learning class were categorised as good and very good in thinking abilities (scores: 2.95-3.74). The traditional class, however, had poor and fair levels of thinking skills (score: 1.14-2.45). On the average, the holistic score of the experiential learning group was at a good level (score of 3.41), while the traditional group was at the fair level (score of 2.25). The mean score between the two groups were significantly different (P<0.05). Among the six questions, the lowest score was in the abilities to explain the assumptions and procedures and the highest score in fair-mindedly (Table 5-10).

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Table 5-10 Analysis of critical thinking skills from answers to the given questions in the experiential learning and traditional groups.

Critical thinking questions	Scores/Levels[a] (Means ± SD)	
	Experiential learning group	Traditional learning group
1) What are the characters of the microorganisms that support the ability for postharvest diseases control? (Inference ability)	3.73 ± 0.734	2.29 ± 0.632
2) Suppose you disagree with the use of biological control for postharvest diseases. Give reason for your disagreement. (Identify argumenative ability)	3.18 ± 0.532	2.05 ± 0.951
3) What are the weak and strong points of biological control method? (Analysis and evaluation ability)	3.28 ± 0.842	2.30 ± 0.942
4) Can all microorganisms on fruits surfaces protect pathogen infection? Give good reason for your answer. How do you prove your answer? (Explain the assumption and procedure abilities)	2.95 ± 0.646	1.14 ± 0.531
5) Is the biological control of postharvest diseases suitable for the present situation? Give good reason for your answer. (Fair-mindedness)	3.74 ± 0.603	2.38 ± 0.792
6) What is the highlight of this article? (Draw warranted conclusions ability)	3.67 ± 0.728	2.45 ± 0.640
Holistic score	3.41 ± 0.681	2.25 ± 0.714
Number of students	33	31

[a]The levels of critical thinking skills: 1=poor, 2=fair, 3=good, and 4=very good

In addition, each student in the experiential learning group (33 students) was given an open-ended questions related to the real situation, i.e., why do you prefer to use the biological control method instead of chemical method for the planting? Students were allowed to write more than one answers. The frequencies of students' answers were as follows: to improve human health (n=27) and food quality (n=21); to reduce impact on food web (n=16), soil pollution (n=18), water pollution (n=10), and air pollution (n=8); to decrease cost of chemical pesticides (n=4). The

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results suggested that most students were concerned about food quality rather than environmental problems.

5.9.3 Laboratory report of hands-on yeast biological control

The laboratory reports of students in experiential learning group were evaluated by rubric scoring of the eight components (Table 5-11). The results showed highest average score was in the component of introduction (3.43), which presented a concise lead-in to the report. The lowest score was in the attractiveness (2.74), which indicated the legible writing but they had some ill-formed letters, print too small or too large. The parts of introduction, hypothesis, purpose, procedure, discussion and conclusion, and overall quality were accomplishable, including. However, the qualities of the data and results parts were in the developing level because they had minor inaccuracies and/or illegible characters.

Table 5-11 Laboratory report of the experiential learning group.

Criteria	Scores/Levels[a] (Means ± SD)	Quality
Introduction	3.43	accomplished
Hypothesis	3.11	accomplished
Purpose	3.28	accomplished
Procedure	3.01	accomplished
Data and results	2.83	developing
Discussion and conclusion	3.11	accomplished
Attractiveness	2.74	developing
Overall	3.18	accomplished
Max. point in each criteria = 4 Number of student (n) = 33		

[a]The levels of laboratory report quality: 1=beginning, 2=developing, 3 = accomplished, and 4 = exemplary.

5.9.4 Concept map of biological control of plant diseases

Each group of students from both experiential learning and traditional lecture was asked to draw a concept map on biological control of plant diseases to probe their conceptual understanding. In an attempt to create the map, the students in each group shared ideas and discussed extensively. Concept maps of the traditional lecture group generally showed less complex conceptualisation than that of the experiential learning group, which can be seen even by a mere glance at the size of the

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maps. Randomly selected concept maps, one each from the traditional or experiential learning groups are illustrated in Figures 5-18 and 5-19. More elements and linking words and more interrelation of concepts are shown in the map from the experiential group when compared to the traditional group.

Most importantly, concept maps of the experiential learning group showed the desirable characteristics of the teaching-learning process, i.e., the interrelation of concepts. They did not appear as fragments but as parts of a whole, whose interconnections are crucial for understanding the content in its more complex sense, which includes generalisations, abstractions and content disposition by order of relevance and meaning. For example, the concepts of relations with the biological control of plant diseases, agriculture, and environment were clearly demonstrated (Figure 5-19) with details of the sub-links to microorganisms related to plants, pathogens, human and pollutions. An analysis of the relations between the concepts depicted on the maps from experiential group showed that they were better understood than those from the traditional group. Moreover, the traditional group's map showed confusion in concept relationship and the word "inhibit" was often presented to indicate a relationship, while the experiential group's maps indicated relationship by using the terms like "mutualism", "parasitism", "antagonism", and amensalism, etc..

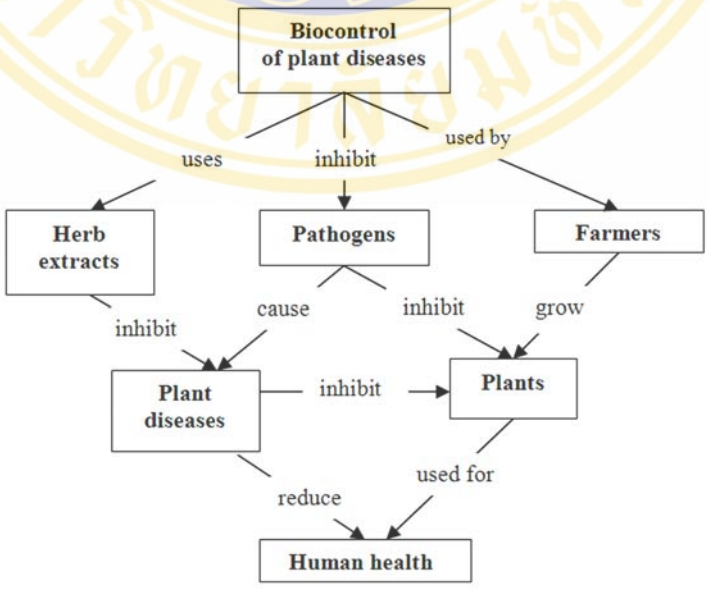

Figure 5-18 Concept map on "biological control of plant diseases" of the traditional learning group.

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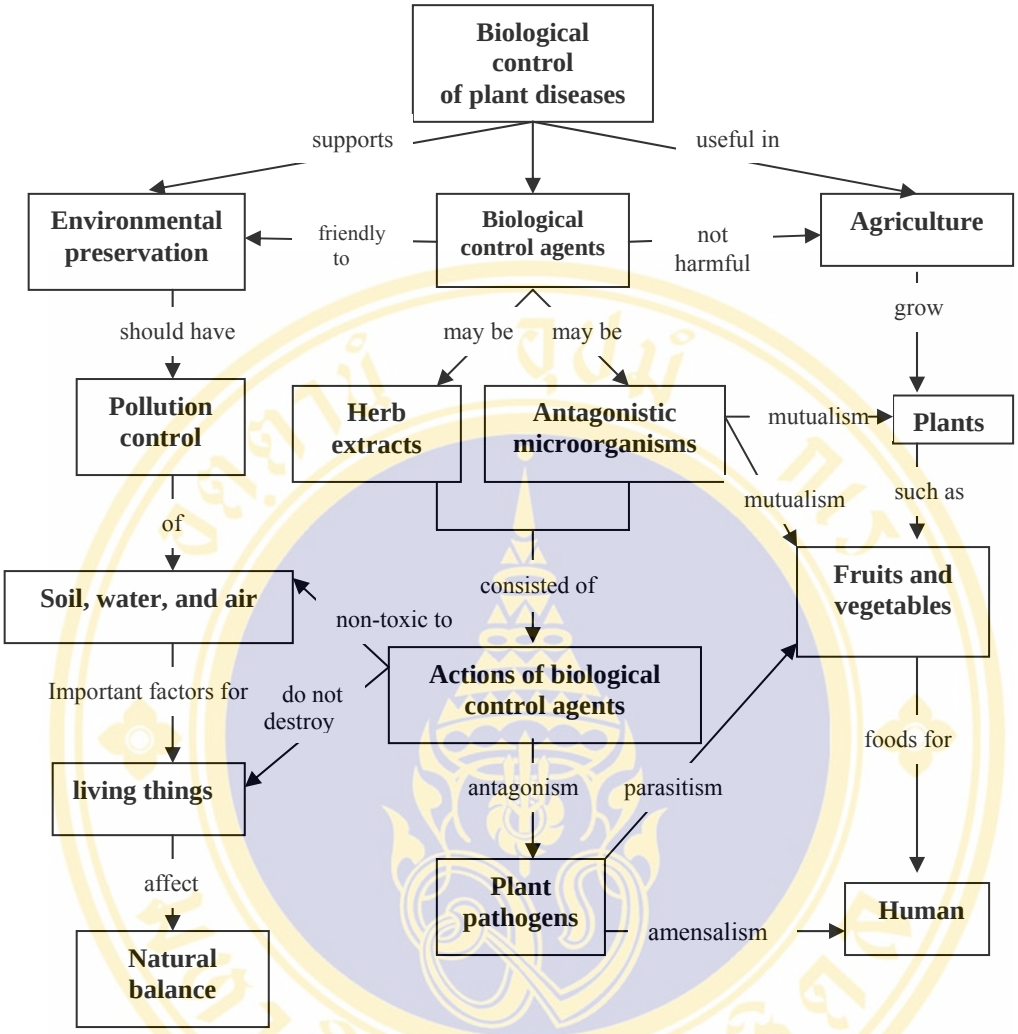

Figure 5-19 Concept map on "biological control of plant diseases" of the experiential learning group.

The average score and qualities of 8 concept maps from the experiential learning group were significantly higher than those from the traditional learning group ($P< 0.05$) as indicated by t-test analysis (Table 5-12). According to the scoring rubric assessment tool of Moni *et al.* (2004), concept maps of the experiential groups showed that most relevant concepts of organism interrelationship in biological control of plant diseases were correct with multiple connections (score 4.39). Understanding of these facts and concepts is demonstrated by a few error links (score 4.01), and the concept map was neat, clear, and legible, has easy-to-follow links and has some spelling errors (score 3.92). In the traditional group, a few relevant concepts of organism interrelationship in biological control of plant diseases were correct with almost no

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connections (score 2.15). Understanding of facts and concepts had some significant errors, and the concept map was neat, legible but with difficult to follow links and some spelling errors (Table 5-12).

Table 5-12 Evaluation of students' concept maps by using scoring rubric (Moni *et al.*, 2004).

Criteria	Scores/Levels (Means ± SD)	
	Experiential learning group	Traditional lecture group
1) **Content:** all relevant concepts of organism interrelationship in biological control of plant diseases are correct with multiple connections (max. 5 points).	4.39 ± 0.781	2.15 ± 0.954
2) **Logic and understanding:** understanding of facts and concepts of organism interrelationship in biological control of plant diseases is clearly demonstrated by correct links (max. 5 points).	4.01 ± 0.921	2.46 ± 1.056
3) **Presentation:** concept map is neat, clear, and legible, has easy-to-follow links and has no spelling errors (max. 5points).	3.92 ± 1.482	2.88 ± 1.045
Total score (max.15 points)	12.32 ± 0.956	7.49 ± 1.067

5.9.5 Interviewing for conceptual understanding

Interviews were used to probe conceptual understanding of students in both experiential learning and traditional lecture groups after completing the teaching sequence. The interviewing questions on organism interrelationship in biological control of plant diseases were divided into three parts to probe each concept (Table 5-13). The results showed that the levels of understanding of students in the experiential learning group were significantly higher than those in the traditional group ($P<0.05$) in the three concepts probed. In the experiential learning class, students were at good and very good levels of conceptual understanding of organism interrelationship and biological control, but the scores of basic concept regarding the modes of action

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between organisms were a little bit lower. Students in the traditional group also showed lesser understanding in the mode of action compared to the other two topics (Table 4-3).

Some answers of students from the experiential group reflected the benefit of the hands-on activities in helping them to answer these questions, for example:

-Well, from my experience in doing the biological control lab, the plant pathogen may be the mold occurring on the fruit sample.

-Yeah, I realise that some microorganisms such as yeasts can be used as biological control agents, from doing this lab.

-I'm sure that the yeast antagonists have to secrete some substances or compete for the nutrients with the plant pathogen, so that they can reduce the growth of pathogens.

-Yeah, to decrease chemical contaminations in agriculture, biological control is the good method for controlling plant pathogens resulting in reduction of the plant diseases as observed in our lab.

During the interviewing process, some students showed enthusiasm that "they would like to do more biological control investigation":

-Teacher, may I test whether the bacteria can inhibit the pathogens on chilli or not? I have learned about its ability from reading some agricultural magazines.

-This biological control experiment is very interesting to me. Ahh, I want to learn how to apply it commercially?

-I'm excited when I saw yeast cells and mold mycelium under microscope or able to culture them to become colonies on plate. Well, I decide to study them more in my science project.

In addition, the students reflected that the supplemented article on biological control of plant diseases helped them understanding biological control and its role in the environment:

-Yeah, microorganisms such as bacteria and yeasts can inhibit growth of the fungal plant pathogens. I realise from reading the assigned article.

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-Ahh, I have read the article and in my view point, biological control method is very important in postharvest because it can reduce chemical contaminations on fruits and vegetables.

-Yes, organisms can inhibit growth of other organisms by competition for nutrients or producing antibiotics and cell-wall degrading enzymes.

Table 5-13 Evaluation of student's answers to the interviewing questions by using scoring rubric method.

Interviewing questions	Probing concepts	Evaluating level/score [a] (Mean ± SD)	
		Experiential learning group	Traditional lecture group
1) When plants were infected by the pathogens, what could be the interrelationships between plants and pathogens?	•Basic ideas concerning the concept of organism interrelationship	3.70 ± 1.245	2.19 ± 1.312
2) What types of relationships are included in this situation?		3.14 ± 1.081	1.39 ± 1.167
3) Please give examples of the plant pathogens?		3.63 ± 1.194	2.57 ± 1.025
4) How does one organism inhibit the growth of another organism?	•Basic concept regarding to the modes of action between organisms	3.01 ± 1.222	1.62 ± 1.298
5) How does the pathogen damage the plant host?		3.11 ± 1.047	1.38 ± 1.391
6) What is the meaning of biological control and its application?	•The concept of biological control	3.75 ± 1.232	2.83 ± 0.928
7) What are the properties of the biological control agents?		3.42 ± 1.098	2.65 ± 1.042
8) Please you give examples of the biological control agents?		3.68 ± 1.103	2.43 ± 0.957

[a]The level/score of conceptual understanding: level 4 (very good) = all answers are clear and accurate, level 3 (good) = all answers are rather clear and understanding, level 2 (fair) = some or all are ambiguous or misconception in the relationship between the given concepts, and level 1 (poor) = the student can not answer all questions.

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5.9.6 Project design

Each group of students in the experiential learning class was asked to apply their knowledge in creating science project as part of their regular course in the next semester. Each group of students presented the proposals after 1 month of brainstorming to the project committees and improved the proposals according to the received comments. They have shown interest in using biological control method in Thai crops and studying the abilities of the biological control agents. The project titles of the eight groups of students are shown in Table 5-14.

Table 5-14 The titles of science projects of students in the experiential learning group.

Group	Title
1	Control of rhizome rot ginger during storage by using antagonistic fungi and plant bio-extracts (Piper Betal leaves and Clove extract).
2	Effects of crude extracts from herb (*Andrographis paniculata*) on bacteria causing soft rot in tomato.
3	Investigation and screening of phylloplane yeasts against fruit rot fungus (*Lasiodiplodia theobromae*) on rambutan.
4	Development of fungal spray (*Cordyceps tuberculata*) for controlling worm of cabbage (*Spodoptera exigua*).
5	Effects of fungal strains isolated from herbivore feces on tomato anthracnose caused by *Colletotrichum capsici*.
6	Selection of antagonistic yeasts from fruits and vegetables for controlling green mold (*Penicillium digitatum*) of postharvest orange.
7	Selection of bacterial strain which inhibits *Pythium aphanidermatum* pathogens of root and basal stem rot of soybean.
8	Screening of *Bacillus* strains for controlling potato wilt caused by *Ralstonia solanacearum*.

These project proposals were evaluated according to content scoring rubric. As shown in Table 5-15, the tasks were ranked as superior in the attributes of objective, variable and control, and hypothesis. However, the procedure, diagram(s), and data interpretation plan were ranked adequate (Table 5-15). The holistic quality of the project proposal was ranked as superior task.

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Table 5-15 The average scores of each project-proposal component by eight groups of students.

Attributes	Mean[a]	SD
Objective	9.12	1.089
Hypothesis	8.10	1.047
Variable and control	8.46	1.194
Procedure	7.05	0.920
Diagram(s)	7.37	0.786
Data interpretation plan	6.83	1.232
Holistic score	8.32	1.057

[a]The score ranges: 0-4 = inadequate task, 5-7 = adequate task, and 8-10 = superior task

5.9.7 Students' attitudes towards biological control experiment

The students' attitudes towards performing the hands-on yeast biological control experiment revealed that most students agreed and strongly agreed with the advantages of hands-on biological control activity with mean scores ranged from 3.79 ± 0.88 to 4.54 ± 0.66 (Table 5-16). Examples of comments from the students are as follows:

-I am lucky to have an opportunity to learn the biological control techniques used in agricultural science.

-The experiment was enjoyable and encouraged good relationship in our group.

-The knowledge from this class can definitely be used in advanced learning of science.

-The experiment promoted awareness of problems from using chemicals in the environment.

-The biology course should have more experiments like this.

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Table 5-16 Students' attitudes towards performing hands-on biological control experiment.

Item	Min.	Max.	Mean	SD
1. This hands-on experiment helps me better understand concepts of organism interrelationship.	3	5	4.17	0.70
2. I have the opportunities to practice microbiological techniques.	3	5	4.54	0.66
3. This hands-on experiment encourages me to think scientifically.	3	5	4.00	0.51
4. This hands-on experiment makes me know better about the microorganisms.	3	5	4.38	0.58
5. This hands-on experiment is interesting.	2	5	4.17	0.76
6. I can apply this hands-on experiment to design science project.	3	5	4.25	0.85
7. This hands-on experiment makes me want to preserve the environment	2	5	3.88	0.80
8. This hands-on experiment makes me think more about the impacts of chemical pesticides to the environment.	1	5	3.79	0.88
9. This hands-on experiment makes me think about the importance of the relationships between organisms and the environment.	1	5	3.96	0.86
10. This hands-on experiment is useful in my daily life.	1	5	4.08	0.97

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CHAPTER VI
DISCUSSION

Scientific Aspect

The part on scientific research demonstrates that four epiphytic yeasts (*P. guilliermondii* strain R13, *C. musae* strain R6, *I. orientalis* strain ER1, and *C. quercitrusa* strain L2) isolated from fruits and vegetables reduce disease incidence in chili pepper fruit caused by *C. capsici* to varying degrees. *P. guilliermondii* strain R13 had the highest biological control efficacy, both *in vitro* and *in vivo* (Figure 5-5, 5-6, 5-7, and Table 5-5). The antagonistic activity of the four yeast strains depends on year, initial concentration and concentration of disease (Figure 5-6). The disease incidence of *C. capsici* infected chili fruits can be reduced to as low as 6.5% by the yeast strain R13, while the other three strains reduce the disease incidence to a lesser extent (Figure 5-6 a, b, c, d). In all four yeast strains, lower disease incidences were observed at the lowest storage temperature tested (Figure 5-7). This is not surprising since the most conducive temperature range for *C. capsici* conidia germination and disease development have been shown to be at 28-33 ^{O}C (Misra and Mahmood, 1960). After harvesting, chili fruits should therefore be kept at a lower temperature to preserve qualities such as colour, texture, and low disease incidence (Agblor and Waterer, 2001). For this reason, a lower temperature range of 18-20 ^{O}C has been used in several studies on the efficacy of various yeast strains (Arras *et al.,* 1999; Droby *et al.,* 1997; Lima *et al.,* 1999).

This most efficacious yeast strain R13 has been successfully identified as *P. guilliermondii* by using the smaller subunit, 5.8S rDNA (ITS1/ITS4), but not by 26S rDNA (D1/D2). This 26S rDNA (D1/D2) taxonomy of *P. guilliermondii* and related taxa, however, may not help differentiate species due to the strong possibility that rDNA D1/D2 sequences and other traits may be too conservative to characterise species within the clade (Suh and Blackwell, 2004). Martorell *et al.* (2005) has suggested that the non-coding internal transcribed spacer regions (ITS) exhibit greater

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interspecific differences than 26S rRNA genes (Cai *et al.,* 1996; Kurtzman,1992), thus allowing the differentiation of closely related species.

Several antagonistic yeasts have previously been isolated from fruits and vegetables and efficaciously used as biological control agents. *I. orientalis* strains 16C2 and 2C2 isolated from grape berry (*Vitis vinifera* L. cv. Negroamaro) were effective in reducing colonisation of *Aspergillus carbonarius* (Bainer) Thom. and *A. niger* Tiegh. on grape berry (Bleve *et al.,* 2006). *C. musae* isolate 18 has been shown to have biological control efficacy against *Botrytis cinerea* Pers.: Fr. infection in strawberries (*Fragaria x ananassa* Duch. cv. Sweet Charlie) (El-Neshawy and Shetaia, 2003). Several strains of *P. guilliermondii* at concentrations similar to those used in this study have previously been shown to have biological control efficacy against infection by various fungi on citrus fruit, grape fruit, apple, pear, table grape and strawberry (Arras *et al.,* 1999; Droby *et al.,* 1997; Lima *et al.,* 1999). However, there is no report on the use of *P. guilliermondii* to control *C. capsici* in chili fruits. This study presents the first evidence that *P. guilliermondii* strain R13 can reduce disease incidence caused by *C. capsici* to as low as 6.5%-6.7% (Figure 5-6 and Table 5-5) in conditions similar to other investigations (Arras *et al.,* 1999; Droby *et al.,* 1997; Lima *et al.,* 1999). Strain R13 helps to delay postharvest decay of intact chili fruits. The disease incidence in R13 treated chili fruits is about two times lower than those of the untreated (Table 5-6). The other yeast strains, i.e., R6, ER1, and L2 although can delay the postharvest decay, but to a lesser extent. The results suggest that *P. guilliermondii* strain R13 has a high potential to be a biological control agent in postharvest control against *C. capsici* infection of chili fruits.

P. guilliermondii has been shown to be nonpathogenic as tested on guinea pigs and outbred Swiss mice (Arras *et al.,* 1999, 2002). Large-scale production of *P. guilliermondii* was efficiently done by using fermentation with inexpensive industrial waste materials (Janisiewicz and Korsten, 2002). The resulting yeasts maintained their antagonistic activity against spore germination of *Penicillium digitatum* (Press.: Fr.). It also effectively inhibited the decay of several citrus cultivars, both injured and non-injured. The degree of postharvest disease prevention by the yeast was comparable to a commonly used wax containing a low concentration of chemical fungicide. In addition, combining the yeast with 200 ppm thiabendazole (TBZ)

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resulted in a reduction in the incidence of decay to a level equal to that of the commercial treatment of 2,000 ppm TBZ. The efficacy of *P. guilliermondii* strain US-7 was maintained under packinghouse conditions at a cell concentration as low as 10^7 cells ml^{-1}, either by drenching or spraying (Droby *et al.,* 1993). These results suggest the possibility of using *P. guilliermondii* strain R13 as a commercial biological control agent for preventing anthracnose disease caused by *C. capsici*.

Postharvest chili fruits are usually preserved by washing or spraying with chlorinated water at 75-400 ppm chlorine and stored at low temperature (7-10 ^{O}C) before shipment (Smith *et al.,* 1998; Suslow, 1997). The present study, however, shows that *P. guilliermondii* strain R13 is more effective in preserving chili fruits than chlorinated water (200 ppm chlorine) at 10 ^{O}C over a period of 30 and 45 days (Table 5-6 and Figure 5-8). This suggests that *P. guilliermondii* strain R13 should replace the chlorinated water for treatment. The preparation of chlorinated water from chorine gas used as such or chlorine arising from hypochlorite salts maybe harmful to the worker (Dänet, 2005). Moreover, the use of chlorine in fruits and vegetables is banned in some countries due to its reaction with organic matter leading to formation of chlorinated compounds: some of their derivatives have been proven to be carcinogenic (Camelo, 2004; Carlsen, 2004). Accumulation of these substances also causes pollution of the environment (Link *et al.,* 1994; Smith, 2001; Stringer and Johnston, 2002). These findings provide a powerful incentive for development of the yeast strain R13 as an alternative to anthracnose disease control by *C. capsici* in postharvest chili fruit. Nevertheless, more studies including field trials need to be performed.

In general, biological control of postharvest diseases of fruits and vegetables is unique in that the antagonists are applied directly to the wounds (the point of entry for the pathogens) and the wound sites are often rich in nutrients. Most postharvest pathogens are necrotrophs, which require nutrients of the dying or dead tissues for germination and initiation of the pathogenic process (Janisiewicz et al., 2000). From microscopic observation, *C. capsici* infected chilli tissue showed necrotrophic growth pattern (Figure 5-10b). The observations indicate that *C. capsici* grows vigorously in wounded chilli fruit, possibly because the fungus produces cell-wall degrading enzymes and a toxin; the fungus appressorium also produces physical pressure that may enhance entrance of the pathogen into the plant cells (Lakshmesha et al., 2005;

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Mehrotra and Aggarwal, 2003). That *Pichia guilliermondii* strain R13 attaches to *C. capsici* hyphae and hyphal tips of germinated spores (Figure 5-11b) agrees with other reports concerning antagonistic yeasts; *Candida oleophila* strain I-182 and *Cryptococcus albidus* (Saito) attached to the hyphal walls of *Botrytis cinerea* and *Monilinia fructicola*, respectively (Chan and Tian, 2005; Saligkarias et al., 2002). *P. guilliermondii* strain 5A and strain US7 have also been shown to attach to the pathogen hypha (Arras et al., 1999; Saligkarias et al., 2002). Protein constituents of cell membrane have been suggested to play an important role in the interaction between yeasts and pathogens through signal recognition between yeast cells and hyphae of pathogens (Chan and Tian, 2005). Moreover, chemical forces such as microbial cell surface charges and other compounds, i.e., mannose residues may also influence the adhesion process (Buck and Andrews, 1999a, b).

The intact or uninjured chilli surface or epidermis which is covered by a wall-defined layer of cuticle can be penetrated directly by a mechanical process preceded by the formation of appressorium at the end of germ tube of *C. capsici* conidia, which exerts a mechanical pressure on the intact wall of the plant host (Manadhar *et al.,* 1995). An infection peg, arising from the apressorium pierces the host cell wall to cause infection. After passing through the chilli cuticle, the infection hypha has to grow through the outer wall of chilli epidermal cell which consists of a network of cellulose microfibrils in a matrix consisting principally of pectic materials (Muruganandam *et al.,* 1991). The infection hyphae of *C. capsici* pass between the radial walls of the epidermal cells without entering them. In the cause of the black spot of chilli fruits (Figure 5-10a), *C. capsic*i hyhpa develops between the cuticle and epidermis and enters the cells of the latter (Louis *et al.,* 1988). The penetration of chilli cell wall relies on enzymatic softening that facilitates the penetration of *C. capsic*i hyhpa. The infected hypha grows quickly through the softened tissues. Pectinase and cellulase from this pathogen play the important role as softening enzymes degrading pectin and cellulose of chilli epidermis layer. The combination of physical pressure by appressorium formation and softening enzymes cause swelling of the invaded cell wall and of cell walls some distance away due to diffusion of the enzymes (Mehrotra and Aggarwal, 2003). Such swellings vary considerably in extent, ranging from sharply

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localised to pronounced thickening which almost obliterates the lumen of the cell (Hamer *et al.,* 1988).

The modes of action of *P. guilliermondii* strain R13 on nutrient competition has been investigated. It is not surprising that *P. guilliermondii* strain R13 competed with *C. capsici* for both sugars and nitrates because competition for sugars has been reported for several other strains. For example, *P. guilliermondii* Y2 competed with *B. cinerea* for glucose and sucrose in infected strawberry leaves (Guetsky *et al.,* 2002). *C. guilliermondii* 3C-1b, F1, and *Metschnikowia pulcherrima* 2.33, 4.4 competed with *Penicillium expansum* and *B. cinerea* for several nitrates (Piano *et al.,* 1997; Scherm *et al.,* 2003). Researchers have suggested that attachment of yeast antagonists to the hyphae of pathogenic fungi may enhance nutrient competition as well as interfere with the ability of the pathogens to initiate infection (El-Ghaouth *et al.,* 1998; Janisiewicz and Korsten, 2002).

Like other antagonistic yeasts, *P. guilliermondii* strain R13 can produce hydrolytic enzymes, and our results demonstrated β-1,3-glucanase and chitinase activity in both solid and liquid media. In addition, the specific activities of β-1,3-glucanase and chitinase from *P. guilliermondii* strain R13 cultured with CWP from *C. capsici* were 2.0 times and 1.1 times higher than those of *P. guilliermondii* strain US7 grown on CWP from *B. cinerea* (Castoria et. al., 1997; Wisniewski *et al.,* 1991). However, the specific activities of β-1,3-glucanase and chitinase were 3.0 times and 1.5 times lower than those of *C. oleophila*, the commercial yeast antagonist (Saligkarias *et al.,* 2002). Chitinase and β-1,3-glucanase hydrolyse fungal cell walls and inhibit the *in vitro* growth of several pathogenic fungi (Schlumbaum *et al.,* 1986; Sela-Buurlage *et al.,* 1993). It is possible that these hydrolytic enzymes play an important role in degradation of the *C. capsici* cell wall, especially when the yeast attaches to the pathogen hypha. This intramural release of the hydrolytic enzymes of *P. guilliermondii* strain R13 suggests similar modes of action to other yeast antagonists including the commercial ones (Chan and Tian, 2005; Droby, 2006; Saligkarias *et al.,* 2002). In addition to degrading hyphae, chitinase and β-1,3-glucanase may help induce resistance of plants to pathogenic fungi (van Loon *et al.,* 1998).

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The results in this study clearly shows that β-1,3-glucanase and chitinase secretions depend on type of carbon source, and cell wall of fungal pathogen induce the highest production of both enzymes. This is in agreement with other findings, in which β-1,3-glucanase activities from *Pichia guilliermondii* isolate 87, *Trichoderma harzianum* and *Pichia anomala*, grown on media supplemented with fungal cell walls, are higher than in media containing glucose (Elad *et al.,* 1982; Jijakli and Lepoivre, 1998; Wisniewski *et al.,* 1991). The lowest activities of β-1,3-glucanase and chitinase in glucose supplemented medium may be due to catabolite repression. Since secretion of β-1,3-glucanase is down regulated in many fungi when glucose is the main source of carbon (Giczey *et al.,* 2001). In several plant-pathogen interactions, the induction and accumulation of chitinase and β-1,3-glucanase, however, are often correlated with the onset of inducing plant resistance (van Loon *et al.,* 1998).

Although the modes of *Pichia guilliermondii* strain R13 is similar to other antagonistic yeasts in nutrient competition and hydrolytic enzyme production, however, it fails to produce killer toxin against the fungus in the condition similar to other studies. This is different from some species of *Pichia*, i.e., *P. anomala*, *P. burtonii*, *P. farinose*, and *P. membranifacians* which produce killer toxin against the molds, i.e., *Penicillium roqueforti*, *P. camembertii*, and *Aspergillus nidulans* (Druvefors, 2004). Killer toxins are mainly produced and active under acidic conditions and the activity decreases with increasing pH and temperature conditions (Sawant *et al.,* 1989). The main argument against the role of killer toxin is that most killer proteins are only active within a very narrow, temperature and pH range. Therefore, it complicates the application in many biological control systems. The pH in the environment would not favor toxin production and the highly variable temperatures would destroy killer toxins (Druvefors, 2004; Suzuki and Nikkuni, 1989).

A number of yeast antagonists have been shown to be effective biological control agents from laboratory to large-scale studies. Multiple modes of action, i.e., directly attaching itself to the pathogens, secreting cell wall degrading enzymes, and competition for nutrients make these yeasts a stronger candidate for biological control than antagonists with only a single mode of action (Wszelaki and Mitcham, 2002). While these agents can reduce postharvest decay, their safety should also be the main

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concern. The high effectiveness of *Pichia guilliermondii* strain R13 in controlling postharvest disease of chilli fruit caused by *C. capsici* (Chanchaichaovivat, 2007) may also be due to the multiple modes of action to the fungal pathogen. Results from this study clearly indicate that *Pichia guilliermondii* strain R13 has multiple modes of action in controlling chilli fruit anthracnose caused by *Colletotrichum capsici*. Our findings confirm previous investigations with other antagonistic yeasts indicating that biological control agents with multiple modes of action seem superior to those with only one mode of action. The information on modes of action is essential for enhancing the chances that this yeast will be successfully used for biological control. For example, understanding mode of action can facilitate registration for commercial use and can be useful for optimizing the formulation and delivery systems. Moreover, the yeast *P. guilliermondii* has been shown to be non-toxic and accepted by many researchers as the biological control agent of many postharvest fruit rots, i.e., apples, grapes, and tomatoes (Arras *et al.*, 1999; Mari and Guizzardi, 1998; Wisnieski *et al.*, 1991). The results suggest that *P. guilliermondii* strain R13 seems to be one of the promising yeast biocontrol agents awaiting for pilot study.

Educational Aspect

In our educational study, the results of research work on yeast biological control of anthracnose disease in chilli caused by *Colletotrichum capsici* (Chanchaichaovivat *et al.*, 2007) have been successfully applied for experiential learning for secondary students to enhance their learning outcomes in a biology course. The developed hands-on activities on yeast biological control demonstrated interactions both *in vitro* and *in vivo* between the plant and two microorganisms: chilli fruit, *C. capsici* and *P. guilliermondii*. A cell study incorporated in the laboratory has been shown to help student understand the different types of cells used in this work. This experiment led to meaningful understanding of organism interrelationships in real life phenomena and enhances high order thinking abilities, especially the scientific and critical thinking ones. It also enabled students to apply the techniques learned for their future investigations and/or in their higher educational experimentation. By using the experiential learning on biological control, the students learning the tasks were rooted centrally into an investigative framework, i.e., performing biological control

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experiment and designing the science projects. In doing the tasks, the students set the questions and get the answers by using scientific process with scientific thinking that could lead to critical thinking. The experiential learning in this study engaged students in authentic real world experiences in a biological control of plant diseases as the starting point for student learning, while the traditional lecture did not provide such an opportunity. In the traditional teaching, the students learn from the teacher without proving their knowledge in the scientific ways or thinking like a scientist, resulting in poor critical thinking.

Results in this study showed that each stage of the learning process according to the four-step model of Kolb and Fly (1975) gained its full value. In step 1, the students gained techniques used in experiment on biological control of plant diseases, which then served as a concrete experience for the subsequent activities. In step 2, the students reflected on what they knew from observation on biological control techniques and then shared the ideas for planning and conducting group experiments. The students attempted to analyse the resulting data and discussed the key points related to the subject matter. Laboratory reports also reflected their understanding and this was used by them to practise presentation of scientific data in the systematic way. In addition, they constructed concept maps (Figure 5-19) which required each student to critically consolidate the knowledge reflecting what they knew. In step 3, the students were able to conclude, generalise and forming the concepts from analysing and evaluating the data (Tables 5-10 and 5-13). The students realised the importance of the biological control context, what the laboratory experience meant to them personally, and what it meant to everyday life from answering a set of questions (Tables 5-10, 5-13, and 5-16). This step enhanced students' high order thinking skills in processing information to construct more sophisticated knowledge or concepts. In step 4, the students expressed what they learned and how they applied to the new situation. For examples, each group of student prepared a proposal for a science project by using conceptual understanding and critical thinking skills from the previous stages and at the same time employed the scientific skills to set the experiment to find answers to the problems. The students participated in the experiential learning cycle in this study gradually accumulated the new knowledge on

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biological control while they attempted to use the scientific process to solve the new problems.

The results in this study clearly indicated a better conceptual understanding in the experiential learning group, compared to the traditional group, as shown by an improvement in students' abilities to formulate answers and explanations of organism relationships in biological control system, i.e., parasitism, antagonism and protocoperation (Table 5-9). They showed the complexity of their knowledge in the concept maps in which various relevant concepts of this learning context were connected, e.g. plant host, microorganisms (fungal pathogen and yeast antagonist), humans, agriculture, natural resources, natural balance, and environmental pollution (Figure 5-19 and Table 5-12). The students in the experiential learning group were able to conceptualise knowledge from the practical work on biological control and showed great interest in what they learned. That was the case supported by a much clearer answer from the interviews and students' curiosity to learn more than what was given in the class (Table 5-13).

Students in the experiential learning group have accomplished the main intended learning outcomes, as reflected by their abilities in pose problems, generating questions, planning and performing the investigations, formulating, communicating, and defending the proposal. This is not surprising since activities in the experiential learning provided opportunities for students to pose their own questions in the scope of biological control of plant diseases and plan their own scientific investigation to test the hypotheses. They dared to interpret, analyse, evaluate the experimental results following the concepts or principles of biological control, and presented their works and shared the ideas with the whole class. They expanded the constructed knowledge from doing biological control laboratory to a more practical work in their science projects (Table 5-14). This is in agreement with previous finding that hands-on experiments increase skill proficiency in science process (Mattheis and Nakayama, 1988). Hands-on activities have also been shown to increase learning achievements in science content (Brooks, 1988). In contrast, the traditional learning group cannot transfer their concepts learned in the lecture situation to a real life phenomenon. This was reflected in their limited connection between concepts of organism interrelationship and the other related concepts such as environmentals impacts from

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these relationships, human life, and natural balance (Figure 5-18), as well as their confused explanations in the interviews. They also lacked experiences in science process skills as shown in their inability to give method for testing antagonists of plant pathogens (Table 5-10). These findings agree with the suggestions of Roberts (1997) and Finn *et al.* (2002) that without the integration of ecological related experiment into secondary education, students often fail to grasp the connection between environmental issues and the science of ecology. Limitation in inquiry-based learning is partly due to the lack of experiment in secondary school ecology curricula (Finn *et al*., 2002).

In the traditional lecture group, most students did not show their interests in the content on organism interrelationship. This teaching style also did not support team work and communication skills, while the experiential learning is both cognition enhancing and motivational by stirring up questions in students' minds that would lead to active learning and group working (Ault, 1986). More importantly, by performing the biological control experiment in this study, the students linked the learning and thinking abilities together as shown in their performances in answering the critical questions and designing their own science projects. The hands-on activities on biological control not only motivated students to learn current course materials but also increased their intrinsic interest in further learning as shown in the interviews (Table 5-13), and improvement in high order thinking (Table 5-10), which is essential for higher education. When students use scientific process to investigate problems, they are at the same time practicing scientific thinking. The experiential learning in this study allowed students to practice scientific process and thinking while they performed biological control experiments and science projects activities. They planned the experiments to find answers for the setting questions. They constructed knowledge from their results and were able to form new questions that lead to further testing. This is the process of scientific inquiry which is very important for science education since students should be able to think like scientists and construct their knowledge from scientific investigation.

As critical thinking is the application of scientific thinking to all areas of study and topics of investigation, if one learns scientific thinking through scientific method at the same time, one can also possess critical thinking (Schafersman, 1997).

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Therefore, it is obvious that the experiential learning on biological control helps students to practice critical thinking; both the hands-on activities and designed science projects have reinforced their critical thinking skills (Tables 5-10 and 5-15). In the laboratory report, they showed good abilities of critical thinking in inferring, analysing and evaluating the data with reasonable discussion. Although the aims of this research work were not to probe the accomplishment of the science projects, but the follow-up study revealed that 90% of the projects succeeded according to the objectives, in spite of some problems e.g. statistical analyses and financial support.

The lower critical thinking skills in the traditional learning group may be due to lack of opportunities to perform the practical work, which is one of the activities that can enhance these thinking skills (Barnes *et al.,* 2003). Moreover, the students were only given the concepts by the teacher without constructing their own. The low critical thinking skills may result in the inability to solve the questions in their real life. The results in this study support the previous findings (Finn *et al.,* 2002; Thomas, 2005) that traditional instruction actually prevents the development of critical thinking skills, which present a rather large problem in secondary education when one considers how frequently these traditional methods are used in biology courses. Therefore, changes of the traditional model instruction will be necessary if the goal is to enhance the critical thinking abilities of secondary students.

Our students had positive attitudes towards the hands-on biological control experiment in many aspects not only in enhancing their conceptual understanding and having skills in science project, but also in increasing awareness in environment preservation (Table 5-16). This was supported by the interviews and answers from the assigned article which showed reasonable and fair-minded ideas on the importance of biological control on agriculture, food, human health, and environmental impact. Moreover, the well-developed science projects on biological control of plant diseases not only reflect their high order thinking ability, but also indicate their attempts to improve the environment and take on social responsibility. The students have shown their interest and enjoyment in doing the experiment as well as looking forward to further investigation. They were excited while observing yeast and fungal cells and asked many questions about microbial habitat, growth, organelles, and their roles in the environment. These conform to the suggestions of Jenkins (1999) that laboratory

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work is important in science education, both for understanding concept and theory and stimulating interests. In addition, the biological control practice has been considered to be of high interest and usefulness by secondary level students and teachers, and has been cited as a top priority topic suitable for the curriculum (Nonnecke, 2001).

This research work suggests that the experiential learning on biological control can possibly be an alternative way for improving students' achievements in biology course. As the biological control is a clean technology not harmful to the environment, and the students should learn how to use it in their daily life. The microbial biological control experiment can be applied to many types of vegetables and fruits and the microorganism antagonists can be either bacteria, yeasts or fungi isolated from natural sources, e.g. soil water or plants making it convenient for students to design various science projects. The teachers in secondary school may adopt and adapt these learning activities as one of the tools to improve learning outcomes in their classes, especially in enhancing students' concept and thinking skills. Hopefully that biological control activity can be used to encourage stewardship ethic of secondary students towards the social, as well as promote them to be critical thinkers required in the all area of higher education.

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CHAPTER VII
CONCLUSION

Scientific Aspect

The possibility of using yeast antagonists isolated from fruits and vegetables to control anthracnose was investigated in order to reduce the use of chemical agents that may be harmful to humans and the environment. Epiphytic yeast strains from 11 species of tropical fruits and vegetables were isolated and identified. Their capabilities to control anthracnose disease caused by *Colletotrichum capsici* were studied. Moreover, the modes of action of the best effective antagonistic yeast (*Pichia guilliermondii* stain R13) against the anthracnose pathogen were investigated. These findings hopefully encourage the use of this yeast antagonist in the field to control anthracnose disease in chilli fruits. The results of this investigation are summarized as follow:

1. Yeast antagonist strains R13, R6, ER1, and L2 isolated from tropical fruits and vegetables were found to inhibit *C. capsici* growth, with bicontrol efficacies of 93.3 %, 83.1%, 76.6%, and 66.4%, respectively.

2. The four effective yeast antagonists (R13, R6, ER1, and L2) were identified to be *Pichia guilliermondii*, *Candida musae*, *Issatchenkia orientalis*, *Candida quercitrusa*, respectively, by using DNA sequencing of 26S rDNA, ITS regions. The results were confirmed by morphological and physiological characteristics.

3. Among the four yeast strains, *P. guilliermondii* strain R13 showed highest efficacy in reducing disease incidence caused by *C. capsici* in chili fruits.

4. *C. capsici* at concentrations of 10^2-10^6 spores ml^{-1} caused lesion diameter between 0.67-1.58 cm on chili fruits and maximum disease incidence was observed at 33 ^{O}C.

5. Optimum concentration of *P. guilliermondii* strain R13 in controlling anthracnose disease was found to be $5x10^8$ cells ml^{-1}.

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6. Postharvest treatment of chili fruits by spraying with 10^8 cells ml^{-1} of *P. guilliermondii* and keeping at 10 ^{O}C for 45 days resulted in 80-90% biological control efficacy with disease incidence of 3.3-16.7%. This is more effective than those conventional with chlorinated water.

7. The colonization of *C. capsici* in chilli wounds caused extensive degradation of host cell walls and altered tissue patterns in chilli wounds. *P. guilliermondii* strain 13 was found attach to *C. capsici* hyphal walls and to restrict proliferation of *C. capsici*, and suppress the disease.

8. The yeast antagonist, *P. guilliermondii* strain 13 was shown to adhere to *C. capsici* hyphae when grew together on chili wound. However, application of only the yeast to the wound site did not cause degradation of host cell walls. They were well preserved and displayed an intense and regular tissue pattern.

9. Studies on competition for nutrients of *P. guilliermondii* strain 13 revealed that the suppression of spore germination and germ tube elongation of *C. capsici* was significantly diminished by glucose, sucrose, and some nitrate sources.

10. The secretions of hydrolytic enzymes as one of the modes of action of *P. guilliermondii* strain 13 was investigated by using plate technique and liquid culture amended with different carbon sources. β-1,3-glucanase and chitinase activities were found in culture with all carbon sources tested. However, both enzymes showed highest activities in *C. capsici* hyphal cell wall rather than laminarin and glucose was the sole carbon source.

11. *P. guilliermondii* strain 13 did not produced killer toxin when cultured under similar condition with other strain tested.

12. The results suggested that tenacious attachment, competition for nutrients, and production of hydrolytic enzymes were the possible modes of action of *P. guilliermondii* strain 13 in suppression of *C. capsici* growth in chilli anthracnose disease.

Educational Aspect

In the educational aspect, the experiential learning on yeast biological control experiment was integrated into biology course of 11th grade students to enhance conceptual understanding of organism interrelationship and to encourage students'

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critical thinking. The effectiveness of this learning model was evaluated in comparison to the traditional lecture. The results of this study were concluded as follows:

1. Results from the scientific part were developed into hands-on activities on yeast biological control for 11th grade students by using the four-mode experiential model of Kolb and Fly (1975) to support students' inquiry based learning. Students can design their own experiment to answer their hypothesis in a scientific way.

2. This experiential learning unit was evaluated for student's learning outcomes, i.e., conceptual understanding on organism interrelationship and critical thinking skills.

3. The experiential learning group was able to accomplish in all four-steps of the experiential model: concrete experience, observation and reflection, forming abstract concept; and testing in new situation steps.

4. The MCQ posttest scores on conceptual understanding in the experiential learning group (score 8.73) was significantly higher than those in the traditional group (score 6.68) at $P<0.05$.

5. An assessment of students' critical thinking skills by holistic critical thinking scoring rubric showed that the experiential learning group was at the good level (holistic score 3.41) while the traditional group was at the fair level (holistic score 2.25).

6. Evaluation of laboratory report on yeast biological control as designed by the students showed that the experiential learning group produced a good-quality report in all criteria, although the part on data presentation was not attractive.

7. Concept maps on biological control of plant disease of the experiential learning group showed more complex conceptualization than those of the traditional group, with more linking words and interrelation between of concepts. The average score of content, logic and understanding, and presentation of the experiential learning group (score 12.32) were significantly higher than those from the traditional learning group (score 7.49) significantly ($P<0.05$).

8. Interviews in the following three concepts: basic concepts on organism interrelationship, modes of action between organisms, and the concept on biological control, showed significantly higher level of conceptual understanding in the experiential learning group when compared to those in the traditional group at $P<0.05$.

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9. Students in the experiential learning class applied their experiences in creating science projects in various topics on biological control. The project tasks were ranked as superior in the attributes of objective, variable and control, and hypothesis, and as adequate in the procedure, diagram(s), and data interpretation. However, the holistic quality of the project proposal was ranked as superior task.

10. The students had positive attitudes towards performing the hands-on yeast biological control experiment. Most students agreed and strongly agreed with the advantages of the hands-on with mean scores ranged from 3.79 to 4.54.

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APPENDIX

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APPENDIX A
HANDS-ON EXPERIMENT OF YEAST BIOLOGICAL CONTROL

Hands-on experiment of yeast biological control was designed for enhancing student conceptual understanding of organism interrelationship. The technique of yeast bioconytol experiment was described as the following.

Introduction

Biological control is the method using one organism including its derivative to control another. During the past decades, this method was developed to be an alternative way to reduce chemical pesticide contamination on foods and in the environment. Recently, biological control of plant diseases is one of the most extensively studied that becoming an important technology. Microbial antagonists (bacteria, yeast, or fungi) that are used as biological control agents of plant pathogen are usually occurring on plant surfaces (Pal and Gardener, 2006). These agents possess a number of important advantages over traditional chemical pesticides which make them commercially attractive. They are considered nonhazardous to humans and animals, biodegradable, and environmentally friendly. They attack specific target organisms, while leaving other beneficial organisms unaffected (Irtwange, 2006).

However, the relationships (interactions) between organisms contributing to biological control of plant diseases are of several types. These interactions can significantly affect plant health in various ways depending on environment. Significant biological control most generally arises from manipulating mutualisms between microbes and their plant hosts or from manipulating antagonisms between microbes and pathogens. The other types of interactions that can have effects on disease control include commensalisms, antagonism, competition, amensalism, and parasitism. Biological control can occur when non-pathogens compete with pathogens for nutrients in and around the host plant. Direct interactions that benefit one population at the expense of another also affect our understanding of biological control. Antagonism can be referred to as amensalism where one species is inhibited,

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typically by toxins or substances produced by the other, which is unaffected. Another interaction that leads to antagonism is parasitism where two unrelated organisms coexist over a prolonged period. The activities of various parasites e.g. yeast antagonists that parasitize plant pathogens result in biological control for plant disease.

With respect to the application of microbial biological control agents, the use of yeasts is quite promising since production of antibiotics probably is not involved in their activity (Irtwange, 2006). Various observations suggest that competition for nutrients between yeasts and molds and parasitism are likely to be the main mechanisms of action. Some examples of yeast isolates use as biological control agents include *Pichia guilliermondii* against chilli anthracnose caused by *Colletotrichum capsici* (Chanchaichaovivat *et al.*, 2007) and *Saccharomyces cerevisiae*, a biological control agent against Botrytis storage rot in many species of fruits and vegetables (Cook *et al.*, 1999) and

In general, the curriculum of biology in high school level desires students develop an understanding the concept of interrelationships between organisms. However, Thai high schools still lack of effective hands-on experiments which enable students directly to study the relationships between organisms in their natural habitat. Only lectures or “paper activities” are used in class. Consequently students may be confused when they are required were assigned to describe or explain the types of the organism interrelationships. They can not give an example or classify types of relationships that occur in real life or imagine what is happening between organisms that live together in the same habitat. Moreover, they do not know why this knowledge is important for their life or useful for their further study. In order to enhance their understanding and motivate creativity of this context, an easy hands-on laboratory, yeast biological control of plant disease, was designed for Grade 11 students. This model experiment provides opportunities for the students to experience biological control of plant disease techniques and group working.

Methods of the experiment

The experiment was carried out by eight groups of Grade 11 students in a science school in Thailand as part of the biology course. This experiment poses

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minimum risks to health while enforcing good laboratory practice including aseptic technique as well as enhancing scientific thinking skills.

1 Plant and microbe samples

1.1 Fungal pathogen (*Collectotrichum capsici*) which causes anthracnose disease in many species of fruits and vegetables was obtained from the Mycological Laboratory of Department of Agriculture (DOA), Thailand. The fungal pathogen can be maintained on potato dextrose agar (PDA) slants at 4 ^{O}C.

1.2 Yeast antagonist, *Pichia guilliermondii*, the species most commonly used as biological control agent (Janisiewicz, 2002) is used in the study. This yeast strain is available from National Collection of Yeast Culture (NCYC, UK) or from Microbiological Resources Centre (MIRCEN, Thailand). *P. guilliermondii* is maintained on PDA slants at 4 ^{O}C.

1.3 Red chili fruit (*Capsicum annuum* L. var. *acuminatum* Fingerh.) was obtained from the market in Nakornprathom Province, Thailand. Three intact fruits with no wound or scar were used in each treatment. Student may use carrot, tomato, onion or apple or others instead of chili fruit in the laboratory, if they wish.

2. Cell observation

Students are asked to observe similarities and differences in cell structure of plant, mold, and yeast under the microscope. The students should prepare their own slides as follows.

2.1 Preparation of plant cell slides

The chili peels are cut into thin pieces by using razor blade. A wet mount of the thin chili peels are made and observed by using the low power objective of your microscope. The length of a chili cell is then estimated using the micrometer. The cell shape and the organelle which are visible cell structures are observed and labeled.

2.2 Preparation of mold and yeast cells slides

The colonies of *C. capsici* and *P. guilliermondii* cultured on PDA plates for 72 h are observed and a pressure-tape preparation of the mold culture using the following steps (Figure 1): (a) A drop of 0.3 % methylene blue was placed on the center of a slide, (b) A piece of clear tape with sticky side down is held in a U-shape, (c) The surface of a fungal colony is touched gently with the sticky side, (d) The sticky

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side of tape is then placed in a drop of methylene blue, (e) The extra length of tape is then folded around edges of slide. These slides of fungi are examined under light microscope for the structures of mold hyphae and yeast cells including conidia, septate hyphae, buds, and vacuoles.

Figure A-1 Pressure-tape preparation of fungal slide.

3. Biological control assay of the antagonistic effect of yeast by an *in vitro* test

P. guilliermondii is investigated for its capabilities for inhibit the growth of *C. capsici* on agar plates. To prepare the agar assay plate, molten PDA plus 15% (w/v) chili juice are poured into a sterile plate, and allowed to solidify for 20 min. Then a 0.6 mm well is made in the center of a plate by using sterile cork borer (0.6 cm in diameter). Yeast and mold are cultured in PDA slants at 28 ^{O}C for 48 h and 72 h, respectively. Cell and spore suspensions are made by adding 5 ml sterile distilled

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water (DIW) into the cultures and the sterile loop are used to suspend the cells and spores.

To test for biological control efficacy, 20 µl of yeast cell suspensions at various concentrations ($5x10^6$, $5x10^7$, $5x10^8$, and $5x10^9$ cells/ml sterile distilled water as counted by hemocytometer) are injected into the wells of plates and kept for 1-2 h to allow penetration of the cell suspensions into the wells. Then the spore suspension of *C. capsici* (20 µl of $5x10^4$ spores/ml) is applied to the well. All plates are incubated at 28 ^{O}C for five days and observed for the fungal growth. Diameters of fungal colonies are recorded as the average length in the x-axis and y-axis. All treatments are performed in triplicate.

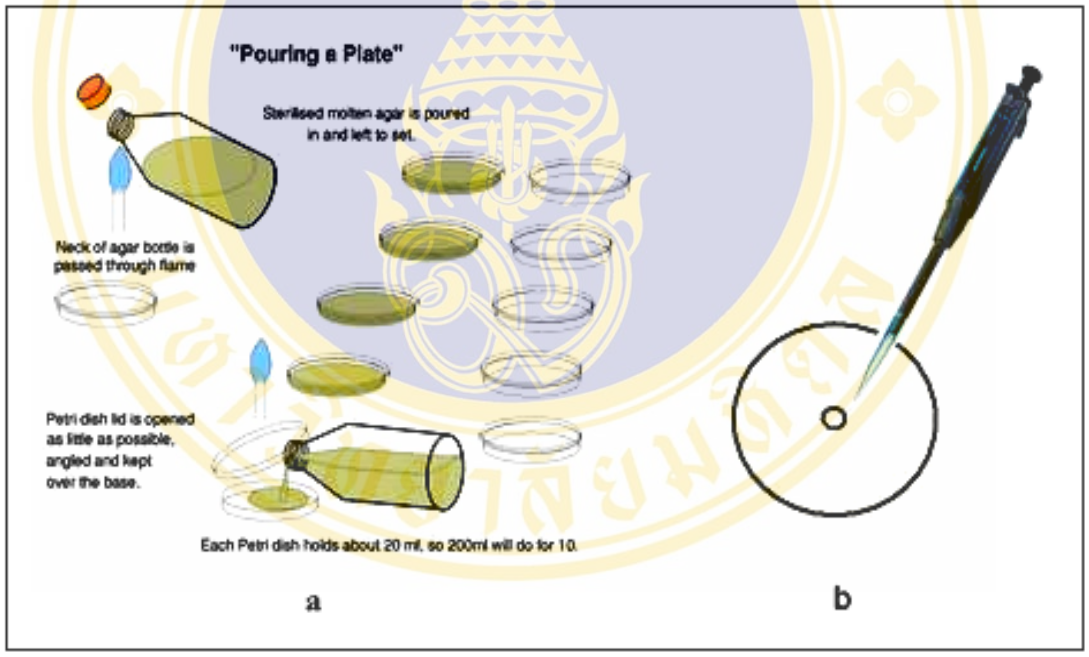

Figure A-2 The preparation of PDA plates by aseptic technique, pouring method (a) and the inoculation sample in to the hole of plate medium by a micropipette (b).

4. Biological control assay by *in vivo* test

Chili fruits (no wound or scar visible on the surface) from untreated orchards. They are surface-sterilized with 0.5% NaCl for 5 min and then washed with tap water. After air drying, the chili fruits are treated with 70% ethanol and wounded by using a sterile cork-borer (0.6 cm in diameter and 1 mm in depth), one wound per

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fruit. Yeast cell suspension (20 µl of $5x10^6$, $5x10^7$, $5x10^8$, and $5x10^9$ cells/ml) are applied to the wounded chili fruits. After 1-2 h to allow the yeast cell to penetrate into the wounds, 20 µl of *C. capsici* ($5x10^4$ spores/ml) are applied to the wounds. The chili fruits are put into sealed plastic bags (one fruit per bag) and stored at 28 ^{O}C in the dark. The severity of disease as indicated by increased wound diameter (lesion diameter) is recorded 5 days after storage. Two control fruits are inoculated with *C. capsici* or *P. guilliermondii* only. Lesion diameter of chili fruit is the average length of lesion in the x-axis and y-axis. Five chilli fruits per treatment are examined in triplicate.

5. Competition for nutrients

Cell suspension of *P. guilliermondii* (50 µl of 5 x 10^9 cells/ml) and spore suspension of *C. capsici* (50 µl of 5 x 10^4 spores/ml) are inoculated in the sterile tubes containing 2 ml of 15% chili juice plus 0.1%, 0.5%, and 1.0% glucose or sucrose. The control tube contains only *C. capsici* in 2 ml of chili juice. All tubes are incubated for 16 h at 28 ^{O}C. Then at least 50 spores per replicate are observed microscopically to determine percentage of spore germination. All treatments are performed in triplicate.

6. Data Analysis

Data analysis of colony and lesion diameter, and percentage of spore germination are done by using the least significant difference (LSD) test of SPSS software (version 10.0 for Windows, SPSS Inc., Chicago, Illinois, USA). The significance at $P < 0.05$ is used for mean separation.

Expected results

The students are asked to record the results in the provided answer sheet.

1. Cell study

Under microscopic observations, draw cell morphologies of red chilli fruit, *C. capsici*, and *P. guilliermondii* with labeled cell structures on the circle frames of Figure A-3.

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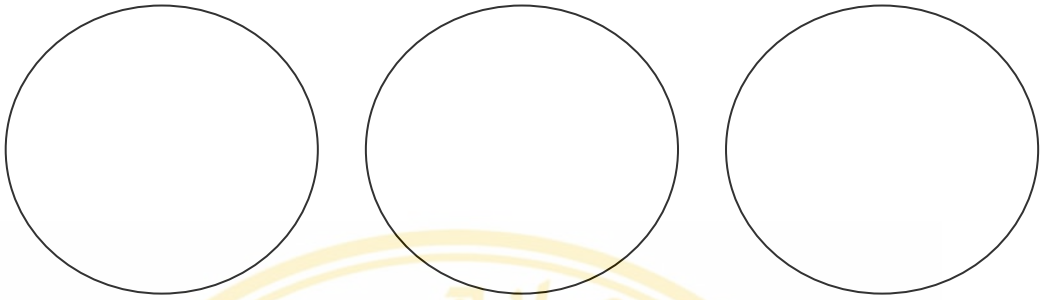

Red chilli fruit (a) *C. capsici* (b) *P. guilliermondii* (c)

Figure A-3 Morphologies of organisms' cells observed by microscope; red chilli fruit (a), *C. capsici* (b), and *P. guilliermondii* (c).

2. Effects of yeast concentrations on control of *C. capsici* growth

Record diameters of *C. capsici*'s colony and lesion of chilli wound at various yeast cell concentrations in Table 1.

Table A-1 Effectiveness of the yeast concentrations on reduction of *C. capsici* colony and lesion diameters of infected chili fruits.

Yeast concentration cells/ml	Colony diameter (cm)	Lesion diameter (cm)
Control		
$5x10^6$		
$5x10^7$		
$5x10^8$		
$5x10^9$		

3. Competition for nutrients between *C. capsici* and *P. guilliermondii*

Record the percentage of spore germination of *C. capsici* at various sugar concentrations in Table 2.

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Table A-2 Effects of sugar (glucose and sucrose) concentrations on ability of *P. guilliermondii* (antagonist) to inhibit spore germination of *C. capsici*.

Treatment	Spore germination (%)	
	Glucose	Sucrose
Inoculated juice + antagonist		
Inoculated juice + 0.1 % sugar + antagonist		
Inoculated juice + 0.5 % sugar + antagonist		
Inoculated juice + 1.0 % sugar + antagonist		
Control (only mold inoculation)		

Critical questions

1. What are the differences of *P. guilliermondii, C. capsici* and chilli fruit cells?

2. What are the roles of *C. capsici* spores and buds of *P. guilliermondii*?

3. What are the interrelationships between *P. guilliermondii*, *C. capsici* and red chilli fruit?

4. How *P. guilliermondii* is able to decrease colony diameter of *C. capsici*?

5. How *P. guilliermondii* is able to decrease lesion diameter of chilli wound?

Discussion and conclusions

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APPENDIX B
CONCEPTUAL TEST

The multiple choice questions (MCQs) and the level of difficulty based on Bloom's Taxonomy (1956) are used to evaluate conceptual understanding of students as shown in Table B-1 and Table B-2.

Table B-1 MCQs for pretest and post test**.**

Item	Level of difficulty[a]
1) What is the result from the relationship between organisms? A. Competition between the organisms B. Survival of organisms C. Balance amount of organisms D. Destruction of natural resources	Analysis
2) What is the character of commensalism? A. Mistletoe on tree B. Orchid on tree C. Lichen on tree D. Flower and insect	Knowledge
3) In the bird flu disease, why human-to-human contact is possible? A. Virus can adapt to live in human body. B. Virus can compete with other microorganisms in the human intestine. C. Human has immunological deficiency for bird flu disease. D. Virus can multiply in all organisms.	Application
4) What is a proverb (motto) from group living of ant and bee? A. Willing to do hard work B. To be on time C. Don't exploit each other D. Unity is important	Synthesis

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Table B-1 MCQs for pretest and post test (continued).

Item	Level of difficulty[a]
5) What is the advantage of coexistence between bird and tree? A. Increase plant resistance to pathogen B. Help plant proliferation C. Balance in plant population D. Reduce environmental pollutions	Evaluation
6) Which is the relationship that plays an important role in population control and natural balance? A. Competition B. Predation C. Saprophytism D. Commensalism	Comprehension
7) In nature, certain bacteria in worm produce chemical that can degrade cellulose, which is the worm food. Therefore the worm obtains more sugar nutrient from bacteria, while the bacteria gain nutrient from worm's metabolism. These organisms are not harmful to each other. What is relationship between the bacteria and worm? A. Protocooperation B. Mutualism C. Commensalism D. Saprophytism	Analysis
8) Studies on 2 types of bacteria in human intestine, *Escherichia coli* and *Streptococcus feacalis* can convert substance, A to C as follows: *S. feacalis* *E. coli* A → B → C What is the relationship between *S. feacalis* and *E. coli*? A. Protocooperation B. Mutualism C. Commensalism D. Saprophytism	Analysis

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Table B-1 MCQs for pretest and post test (continued).

Item	Level of difficulty[a]
10) What is the defect of cohabitation? A. Take different responsibility B. Increase pollutions in the environment C. Increase migration of organisms D. Competition for resources	Evaluation
11) What is the advantage of biological control by mold, *Trichoderma* spp., against the pathogen, *Fusarium* spp. in plant? A. Contaminations of chemicals in soil B. Use of inorganic fertilizers in the soil C. Increase of predators in the environment D. Plants are destroyed by fungal pathogen	Application
12) What is not the principle of biological control? A. Control by producing inhibitory substances B. Big organisms control small organisms C. Control by parasitism D. Control by competition for nutrients	Comprehension
13) What is the obvious defect of chemical treatment in the agriculture? A. Contamination of toxic substances B. Low quality of agricultural products C. The boycott of the export D. The incomes of farmers are not consistent	Evaluation
14) What is the character of the antagonist for biological control of plant diseases? A. It is in the group of microorganisms closely related to plant pathogen. B. It is a pesticide resistance strain C. An organism which can proliferate and occupy the space faster than the pathogen. D. It must be a bacterial strain	Comprehension

[a]Level of difficulty of tested items were classified according to Bloom's Taxonomy.

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Table B-2 Level of difficulty based on Bloom's Taxonomy**.**

Category	Level	Definition
Knowledge	Lowest point value	Requires that the student recalls data or information directly. Memorized facts.
Comprehension		Requires that the student understands the meaning, translation, and interpretation of instructions and problems. States a problem in one's own words.
Application		Student uses a concept in a new situation. Applies what was learned in the classroom into novel situations.
Analysis		Separates material or concepts into component parts so that its organizational structure may be understood. Demonstrates a students' ability to distinguish between facts and inferences.
Synthesis		Student builds a structure or pattern from diverse elements. Puts parts together to form a whole, with emphasis on creating a new meaning or structure.
Evaluation	Highest point value	Make judgments about the value of ideas or materials.

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APPENDIX C
ARTICLE AND CRITICAL THINKING QUESTIONS

The activities of reading biological control article “Biological Control of Postharvest Diseases” and answering the critical questions are shown in Table C-1.

Table C-1 Article and critical thinking questions.

Task	Critical thinking skills	Time (min)
Read the article biological control of postharvest diseases: The chemical treatment of postharvest diseases at present causes many problems according to the resistances of pathogens and pollutions in the environments. Moreover, the restricted permission of chemical treatment in the agricultural sector is increasing; for example, benomyl that has been used widely in Thailand, but now it is not allowed to use in Japan and USA. In the future, it is difficult to synthesize the new chemical that is safe for human and the environment because of the high cost of investigation and the health consideration. In addition, there is increasing demand for healthy food without chemical contamination. Therefore, the alternative method to control postharvest diseases is needed. Besides the temperature treatment and atmosphere control, biological control is the promising method; especially the use of antagonistic microorganisms such as bacteria, yeasts, and mold. The application of microbial antagonists depends on their abilities to grow faster than the pathogen, thus they can remove nutrients and occupy the space from the pathogen. Application of biological control agent in the field to control postharvest decays of fruits is used in 2 ways. First, the antagonists developed for postharvest application are applied	Reading critically	8

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Table C-1 Article and critical thinking questions (continued).

Task	Critical thinking skills	Time (min)
just before harvest. In order to precolonize the fruit surface with an antagonist immediately before harvest, so that wounds inflicted during harvest can be colonized by the antagonist prior to colonization by a pathogen. In the other approach, antagonists, selected for field application, are applied throughout most of the fruit development, to reduce latent infections that can originate as early as bloom time and cause fruit decay after harvest when the natural mechanisms of resistance have broken down. The antagonists are often isolated on the surface of plants; this natural presence makes them more likely to succeed because of their colonization ability and environmental adaptation. The examples of antagonist applying in postharvest disease control are: 1. Yeast strains on Israeli orange are able to preserve postharvest oranges. They found that when the oranges were washed by water, the washed water contained a lot of the yeast strains with a little of fungi. If the orange is washed after harvesting, the fruit will decay rapidly. This indicates that the yeast strains can delay the fruit rot from fungal pathogen growth. 2. The use of yeast *Pichia gulliermondii* to control fungal pathogen, *Penicillium digitatum*, in grape fruit by competition for nutrients and space. In addition, this yeast is able to produce hydrolytic enzymes capable of attacking the cell wall of fugal pathogen, thus the pathogen is unable to proliferate on the grape fruit. 3. The use of bacteria *Bacillus subtilis* to control postharvest disease of peach caused by *Monilinia fructicola*. This bacteria produce antibiotic that can inhibit spore germination of fungal pathogen; therefore, *M. fructicola* is unable to future proliferate on the peach fruit.		

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Table C-1 Article and critical thinking questions (continued).

Task	Critical thinking skills	Time (min)
However, it is necessary to study the survival of antagonist on the postharvest product besides the ability to control the disease because the product was treated after harvesting such as the use of low temperature and detergent involvement. Moreover, it should consider the wound infection by other pathogens that can grow replaceable.		
Answer the critical questions:		
1) What are the characters of the microorganisms that support the ability for postharvest diseases control?	- Inference	3
2) Suppose you disagree with the use of biological control for postharvest diseases. Give reason for your disagreement.	- Identify the arguments	3
3) What are the weak points and strong points of biological control method?	- Analysis and evaluation	3
4) Can all microorganisms on the surfaces of fruits protect the pathogen infection? Give good reason for your answer. How do you prove your answer?	- Explain (assumptions and procedures)	3
5) Is the biological control of postharvest diseases suitable for the present situation? Give good reason for your answer.	- Fair-mindedly	3
6) What is the conclusion of this article?	- Draw warranted conclusions	4
7)* Why do you prefer to use biological control method instead of chemical method for the planting? (*The question is used for evaluation the attitudes of students toward the biological control method)	- Evaluation	3
Total		30

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BIOGRAPHY

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