DISCUSSION

In this study, the new semi-selective medium (XSC) proved to be effective for growing Xsc cultures and isolating Xsc from citrus samples because of its high recovery and differentiating capacity. XSC medium also uses commonly available ingredients, inexpensive and easy to prepare.

Most bacterial and/or fungal contaminants from citrus samples were suppressed by XSC medium. Xsc was recovered from all citrus samples by XSC medium (Table 7) and rapid determination by eye can be done within 2 days after plating. SX and FS media can recover Xsc only one sample and it slower grew on there media that took 3 and 5 days, respectively. The results high inhibition growth of SX and FS media because of they contained too much concentration/number of dyes or antibiotics in recipes.

Two basic dyes, methyl green 15 µg/ml and methyl violet 2B 2.5 µg/ml, were added to improve saprophyte inhibition and colony differentiation during starch hydrolysis to enhance medium selectivity. These dyes were generally used in several semi-selective media of xanthomonads (Schaad *et al*, 2001). Fung and Miller (1973) showed that many gram-positive bacteria did not grow in the presence of 10 µg/ml of methyl green or methyl violet B. Methyl violet 2B was more growth inhibition of Xsc than methyl green in this study.

Antibiotics in XSC medium were used cephalexin 25 µg/ml and cycloheximide 50 µg/ml to eliminate saprophytic organisms. Cephalexin specifically inhibited the yellow bacterial saprophytes, *Erwinia herbicola* (McGuire *et al*., 1986). Cycloheximide was more effective to inhibit growth of saprophytic fungi (McGuire *et al*., 1986).

New specific primers, 354 primers were designed from a fragment in chromosomal DNA of Xsc that translated to conserved hypothetical protein. The results of 354 primers showed specific amplify DNA of all strains of Xsc that isolated

from different hosts and geographical areas in Thailand and strains from Japan and Saudi Arabia by giving expected 354 bp PCR fragment but not from other xanthomonad (Table 9). This is the first report of using sequences from a conserved hypothetical protein in chromosomal DNA to design specific PCR primers for detection of Xsc. The PCR primers from conserved hypothetical protein region showed specificity than primers from plasmid DNA (VM3-VM4 and 2-3 primers, Table 9).

The primers designed from chromosomal DNA, KF-KR, had specific amplification with all strains of Xsc in experiment. The primers also produced prominent band with Xsc A and A*-strain but the reaction with A^{w}-strain and *X. fuscans* subsp. *aurantifolii* (B and C-strains) were inconsistent and also gave more primer-dimer products (Mavrodieva *et al.*, 2004). At present, PCR product fragment of KF-KR primers still cannot be indentified when searching with Genbank database by using BLAST program provided by National Center for Biotechnology Information (NCBI).

For the primers designed from plasmid DNA, 2-3 primers and *pthA* gene family and VM3-VM4 primers, amplified not only all strains of Xsc but also with other xanthomonad strains. Primers 2-3 cross-reacted with *X. smithii* subsp. *smithii* and VM3-VM4 cross-reacted with *X. fuscans* subsp. *aurantifolii*, *X. smithii* subsp. *smithii* and *X. campestris* pv. *glycines* (Table 9).

The plasmid DNA has been reported as being easily cured, frequently mutants within the internal sequences and not present in all pathogens (Miyoshi, 1998). The propose of VM3-VM4 primers is to develop universal detection of Xsc and *X. fuscans* subsp. *aurantifolii*. However, results in this experiment showed that the primers did not completely detect all target strains of xanthomonad. They detected 1 strain from 3 strains of *X. fuscans* subsp. *aurantifolii* (B-strain), all 4 strains of *X. fuscans* subsp. *aurantifolii* (C-strain) and did not detect any of *X. fuscans* subsp. *aurantifolii* (D-strain). Nine strains of *X. smithii* subsp. *smithii* and *X. campestris* pv. *glycines* also reacted with VM3-VM4 primers. The *pthA*, *pthB* and *pthC*, members of *pthA* gene

family, belong to a family of avirulence or pathogenicity genes found in the genus *Xanthomonas* (the *avrBs3/pthA* gene family) (Gabriel, 1997; Leach and White, 1996). and these may be transferred horizontally on plasmids between Xsc and *X. fuscans* subsp. *aurantifolii* (Brunings and Gabriel, 2003). The results of this experiment also confirmed that *avrBs3/pthA* gene family is distributed in *X. smithii* subsp. *smithii* and *X. campestris* pv. *glycines*.

The assay of 354 primers with classical PCR had ability to detect a lower limit of about 70 CFU/µl of viable cells and the lower limit of detection of 50 pg/µl of purified Xsc total DNA. Sensitivity of other primers to detect viable cells of Xsc and purified Xsc total DNA were 10 CFU/µl and 25 pg/µl for 2-3 primers and 10 CFU/µl and 1 pg/µl for VM3-VM4 primers. The target PCR product of 354 primers located in chromosomal DNA which Xsc carries a single copy per cell, lower than plasmid that Xsc carries multiple copies per cell (Mavrodieva *et al.*, 2004).

Bactericide application is generally practiced for controlling citrus canker and copper compounds are the most popular bactericide. Boonwatana (1991) showed Xsc in orchards that are usually sprayed with copper compounds had higher resistance to copper than Xsc strains from orchards unsprayed with copper compounds. In this study, we chose to evaluate Canoron®, which was previously reported (Lertsuchatavanich, unpublished data) to induce resistance against Xsc in greenhouse for reducing citrus canker symptoms.

In laboratory experiments, the data showed that Canoron® had no effect on Xsc growth *in vitro*. Unlike Phytomycin®, Thianosan® and Cupravit®, which were directly effective in inhibiting growth of Xsc. All Xsc strains were resistant to copper hydroxide (Funguran®) and most of them were resistant to copper oxychloride (Cupravit®) at the recommendation rate. Therefore, it was indicated that resistance of Xsc to copper compounds occurred as previously reported in xanthomonad populations due to regular spraying of copper bactericides (Boonwatana, 1991; Marco and Stall, 1983 and Rinaldi and Leite, 2000).

In greenhouse experiments, artificial infection of lime (*C. aurantifolia*) with Xsc was used to evaluate the efficacy of Canoron® to control bacterial citrus canker. Canoron® FP and WP formulations were more effective on controlling citrus canker disease than Thianosan®, Cupravit®, Masbrane® and Phytomycin® ,which was the most effective control Xsc in laboratory. The mode of action of Canoron® was presumed to be due to induced systemic resistance (ISR) because it had no direct antimicrobrial activity *in vitro* test but had *in vivo* activity on lime which was similar to Actigrad (acibenzolar-S-methyl) and Messenger (harpin protein) (Graham and Leite, 2004). The compound was effective when applied *in planta* by reducing the number of citrus canker lesions and lesion development of citrus canker (Fig. 10). Therefore, this was the first report of Canoron® as ISR compound against bacterial citrus canker.

In field experiments, high intensity rain is known to favor splash dispersal of plant pathogens (Madden, 1992). High intensity rainfall occurred during Experiment 1 (Oct. 7 – Nov. 17, 04) and Experiment 4 (Oct. 12 – Nov. 2, 05) leading to rapid increase of disease incidence (%). The climate conditions throughout Experiment 2 (Nov. 10 – Dec. 22, 04) were low temperature, low relative humidity (%) and little rainfall which were not suitable for disease development, and therefore low disease incidence (%) was observed. These results agreed with the previous reports in a winter season of Argentina and Japan and tropical area of Reunion Island of France that during low temperature the population of Xsc in the lesion decreased (Koizumi, 1977, Pruvost *et al.*, 2002 and Stall *et al.*, 1980).

CONCLUSION

The XSC medium was suitable for isolation of Xsc from citrus materials because the medium was high recovery rate of Xsc similar as NA, the general non-selective medium for bacteria and successful to suppress saprophytic organisms by using 2 general dyes for xanthomonads and antibiotics from SX and FS media. The concentration of dyes and antibiotics were not only suitable to allow Xsc growth but also suppressed saprophytes that supported XSC medium to success in isolation.

In this study, XSC medium show good results in isolation and effectiveness in recovery of Xsc. Thus, XSC medium will be suitable for combined use in BIO-PCR technique in detection of Xsc.

New specific primers, 354 primers gave specific amplification in chromosomal DNA of Xsc in a region of conserved hypothetical protein gene. The results showed that the primers were specific reacted with Xsc only but not cross reacted with other xanthomonads especially in the group of bacterial citrus pathogens including *X. fuscans* subsp. *aurantifolii* (B-, C- and D-strains) and *X. alfalfae* subsp. *citrumelo* (E-strain). Plasmid DNA primers (VM3-VM4 and 2-3 primers) showed cross-reaction to other xanthomonads. The false positive results in detection are very important for quarantine of the important disease like bacterial citrus canker because it will effect on international trade.

The problem of the primers that designed from chromosomal DNA is lower sensitivity than the primers based on plasmid DNA because number of copy of target fragment in chromosomal DNA has only one copy but in plasmid DNA has more than one copy. The sensitivity of 354 primers for detection of Xsc should be improved by Real-Time PCR or BIO-PCR techniques in the future.

From this experiment, Canoron® was an alternative chemical for use in controlling citrus canker disease instead of copper compounds that commonly used as a standard chemical control. Canoron® was not effective to control Xsc in laboratory

as Phytomycin® (antibiotics) and Cupravit® (copper compound). However, in greenhouse, the results were different from laboratory that Canoron® showed effective to control citrus canker. Canoron® was selected to evaluate for control efficacy in field experiment 4 times in 2 years (2004-2005) and results showed inconsistent effective control because of uneven of disease distribution and problem of climate that caused water logging in field experiment areas. In the future, artificial inoculation for even distribution of canker lesions should be done in the experiment.

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APPENDIX

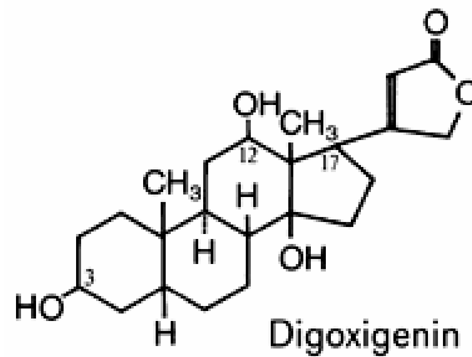

Figure 1A Molecule of digoxigenin

Source: PCR DIG Probe Synthesis Kit, Roche®

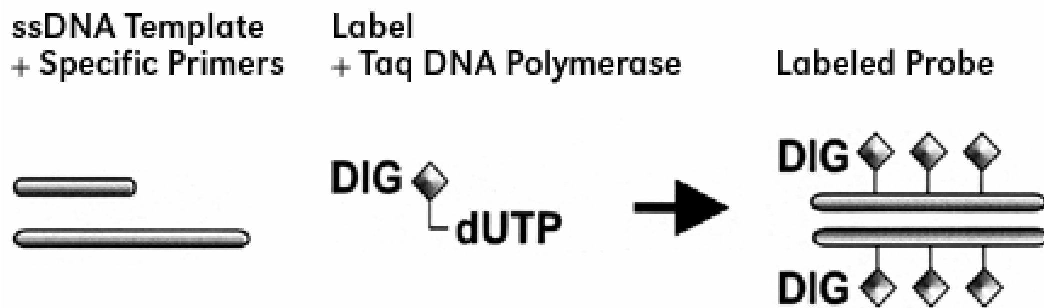

Figure 2A Incorporation of Digoxigenin-11-dUTP to synthesize DNA in a standard PCR reaction.

Source: PCR DIG Probe Synthesis Kit, Roche®

Figure 3A Structure of pCR®8/GW/TOPO® vector (A) and map of ligation site of PCR product of pCR®8/GW/TOPO® vector that show the position of sequencing primers GW1/GW2 (B)
Source: pCR®8/GW/TOPO® TA Cloning® Kit, Invitrogen®

Semi-selective media

SX agar

	Per liter
Starch (soluble-potato)	10.0 g
Beef extract	1.0 g
Ammonium chloride	5.0 g
K_2HPO_4	2.0 g
Methyl violet 2B	1.0 ml*
Methyl green	2.0 ml**
Agar	15.0 g
After autoclaving add:	
Cycloheximide	5.0 ml***

* 1% solution in 20% ethanol.
** 1% aqueous solution
*** Add 5.0 g to 10.0 ml methanol, bring to 100ml with sterile water.

Fieldhouse Sasser (FS) agar

	Per liter
Starch (soluble-potato)	10.0 g
Yeast extract	0.1 g
K_2HPO_4	0.8 g
KH_2PO_4	0.8 g
$MgSO_4$ $7H_2O$	0.01 g*
Methyl green	1.5 g**
KNO_3	0.5 g
Agar	15.0 g
After autoclaving add:	
Cycloheximide	5.0 ml***
Pyridoxine-HCL (1 mg/ml)	1.0 ml
Cephalexin (10 mg/ml)	2.5 ml
Gentamycin (2 mg/ml)	0.2 ml****
Trimethoprim (10 mg/ml of ethanol)	3.0 ml
D-methionine (1 mg/ml)	3.0 ml

* Add first and be sure it is fully dissolved.
** 1% aqueous solution.
*** Add 5.0 g to 10.0 ml methanol, bring to 100 ml with water, and filter sterilize (0.22 µm membrane).
**** Gentamycin can be toxic to some xanthomonads and is often eliminated.