CHAPTER IV
RESULTS

Soil pH analysis

Table 4.1 showed average soil pH of soil samples collected from 15 subdistricts in three districts of Phitsanulok Province. Table 4.2 showed 525 bacterial isolates obtained from root nodules of soybean cultivars separately grown in the collected soil samples, 5 isolated per 1 subdistrict with 1 soybean cultivar. Table 4.3 showed 105 bacterial isolates used in growth studies, RAPD-PCR fingerprinting, and multiplex PCR reactions. There are 105 selected by 1 isolated from 5 isolated in 1 subdistrict with 1 soybean cultivar.

Table 4.1 Determination of pH of soil samples collected in 2005.

Soil collection site	Average soil pH in 0.01M $CaCl_2$*
Sub districts in Chat Trakarn District	
Chart Trakarn	4.56
Pa Daeng	7.72
Suan Miang	4.60
Sub districts in Bang Rakam District	
Bang Rakam	5.84
Pluk Raed	6.67
Pan Sao	5.55
Bung Kok	6.41
Nong Ku-la	6.25
Chum Saeng Songkram	5.43
Bou Thong	5.77
Kui Muang	5.59

Soil collection site	Average soil pH in 0.01M $CaCl_2$*
Sub districts in Prom Piram District	
Wong Kong	6.08
Ta Look Taem	6.08
Wang Won	6.41
Dong Pa Kam	5.54

*** An average of duplicates.**

The soil average pHs indicated all the soil samples were acidic with soils from Chart Trakarn and Suan Miang the most acidic with the average pH 4.56 and 4.60, respectively. Results of further soil analysis were given in Appendix C which also contained information on numbers of soybean growers, soybean productivity, and soybean cultivation areas in the 15 subdistricts of Phitsanulok in 2005.

Table 4.2 Code of bacteria isolated from root nodules of seven soybean cultivars grown in soils from districts in Phitsanulok Province.

Soybean cultivars	Sor Tor 1	Sor Tor 2	Sor Tor 3	Sor Jor 4	SorJor 5	Chiangmai 2	Chiangmai 60
Sub districts in Chat Trakarn District							
Chat Trakarn	D21-D25	D26-D30	D31-D35	D1-D5	D6-D10	D16-D20	D11-D15
Pa Daeng	D91-D95	D96-D100	D101-D105	D71-D75	D76-D80	D86-D90	D81-D85
Suan Miang	D56-D60	D61-D65	D66-D70	D36-D40	D41-D45	D51-D55	D46-D50
Sub districts in Bang Rakam District							
Bang Rakam	D491-D495	D496-D500	D501-D505	D506-D510	D511-D515	D515-D520	D521-D525
Pluk Raed	D246-D250	D251-D255	D256-D260	D261-D265	D266-D270	D271-D275	D276-D280
Pan Sao	D281-D285	D286-D290	D291-D295	D296-D300	D301-D305	D306-D310	D311-D315

Soybean cultivars	Sor Tor 1	Sor Tor 2	Sor Tor 3	Sor Jor 4	SorJor 5	Chiangmai 2	Chiangmai 60
Bung Kok	D316-D320	D321-D325	D326-D330	D331-D335	D336-D340	D341-D345	D346-D350
Nong Ku-la	D351-D355	D356-D360	D361-D365	D366-D370	D371-D375	D376-D380	D381-D385
Chum Saeng Songkram	D386-D390	D391-D395	D396-D400	D401-D405	D406-D410	D411-D415	D416-D420
Bou Thong	D421-D425	D426-D430	D431-D435	D436-D440	D441-D445	D446-D450	D451-D455
Kui Muang	D456-D460	D461D465	D466-D470	D471-D475	D476-D480	D481-D485	D486-D490
Sub districts in Prom Piram District							
Wong Kong	D141-D145	D146-D150	D151-D155	D156-D160	D161-D165	D166-D170	D171-D175
Ta Look Taem	D126-D130	D131-D135	D136-D140	D106-D110	D111-D115	D121-D125	D116-D120
Wang Won	D176-D180	D181-D185	D186-D190	D191-D195	D196-D200	D201-D205	D206-D210
Dong Pa Kam	D211-D215	D216-D220	D221-D225	D226-D230	D231-D235	D236-D240	D241-D245

D = Duangporn Emampaiwong

Table 4.3 Bacterial isolates used for RAPD-PCR fingerprinting.

Soybean cultivars	SorTor 1	SorTor 2	SorTor 3	Sor Jor 4	SorJor 5	Chiangmai 2	Chiangmai 60
Sub districts in Chat Trakarn District							
Chat Trakarn	D24	D28	D35	D3	D9	D20	D11
Pa Daeng	D92	D97	D103	D71	D77	D87	D83
Suan Miang	D57	D64	D66	D37	D43	D54	D48
Bang Rakam	D494	D499	D501	D509	D511	D520	D521
Pluk Raed	D250	D252	D257	D263	D267	D273	D279

Soybean cultivars	SorTor 1	SorTor 2	SorTor 3	Sor Jor 4	SorJor 5	Chiangmai 2	Chiangmai 60
Sub districts in Bang Rakam District							
Pan Sao	D281	D286	D291	D296	D301	D306	D312
Bung Kok	D316	D325	D326	D332	D337	D345	D347
Nong Ku-la	D353	D360	D361	D366	D373	D378	D384
Chum Saeng							
Songkram	D388	D395	D399	D404	D408	D414	D416
Bou Thong	D423	D430	D435	D438	D442	D447	D455
Kui Muang	D459	D464	D467	D473	D477	D481	D490
Sub districts in Prom Piram District							
Wong Kong	D141	D147	D154	D157	D165	D169	D171
Ta Look Taem	D128	D132	D137	D106	D114	D121	D120
Wang Won	D176	D182	D188	D195	D200	D203	D208
Dong Pa Kam	D213	D217	D221	D226	D232	D237	D243

Growth curves of bacterial isolates

Figures 4.1(a) - 4.1 (f) showed growth curves of 105 bacterial isolates grown in yeast extract mannitol broth. The results showed 11 isolates were fast-growers and 94 isolates were slow-growers. Viable plate counts of all the 11 fast-growers and three representatives of slow-growers were shown in Figure 4.2. The plate count results confirmed the fast-and slow- growing nature of the bacterial isolates.

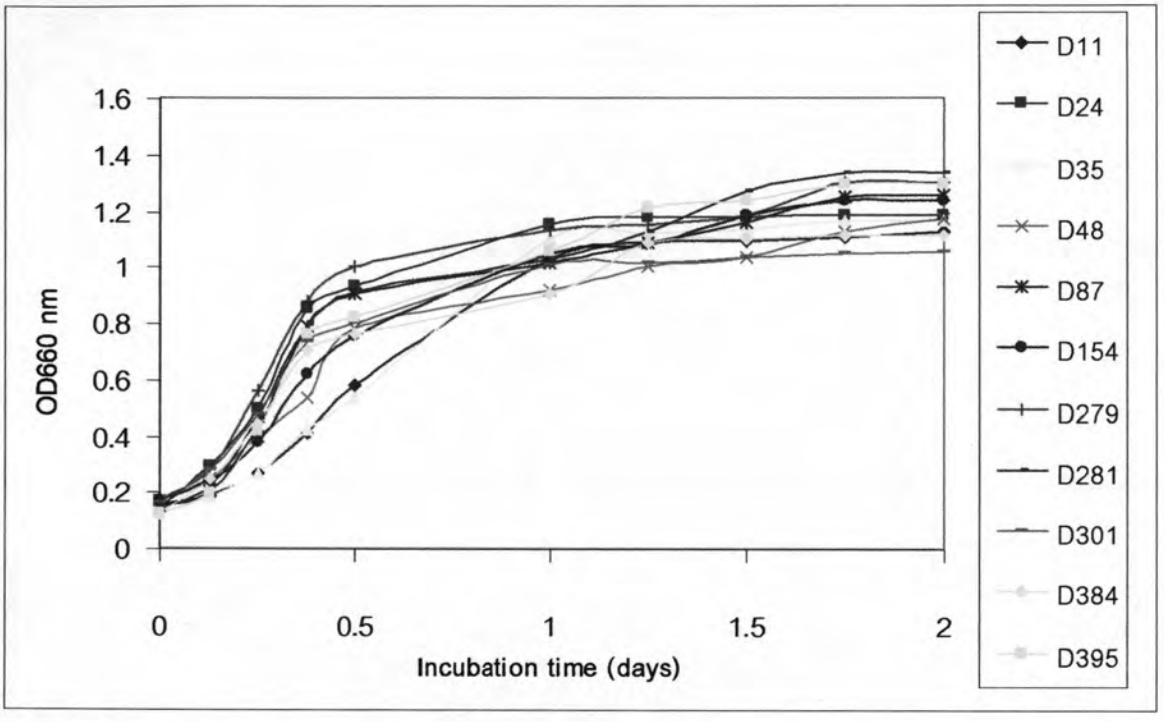

Figure 4.1 (a) Growth curves of 11 bacterial isolates from root nodules of soybean cultivars separately grown in soil samples from Phitsanulok Province. Growth medium was yeast extract mannitol broth. Cell cultures were grown at 200 rpm, 30 °C.

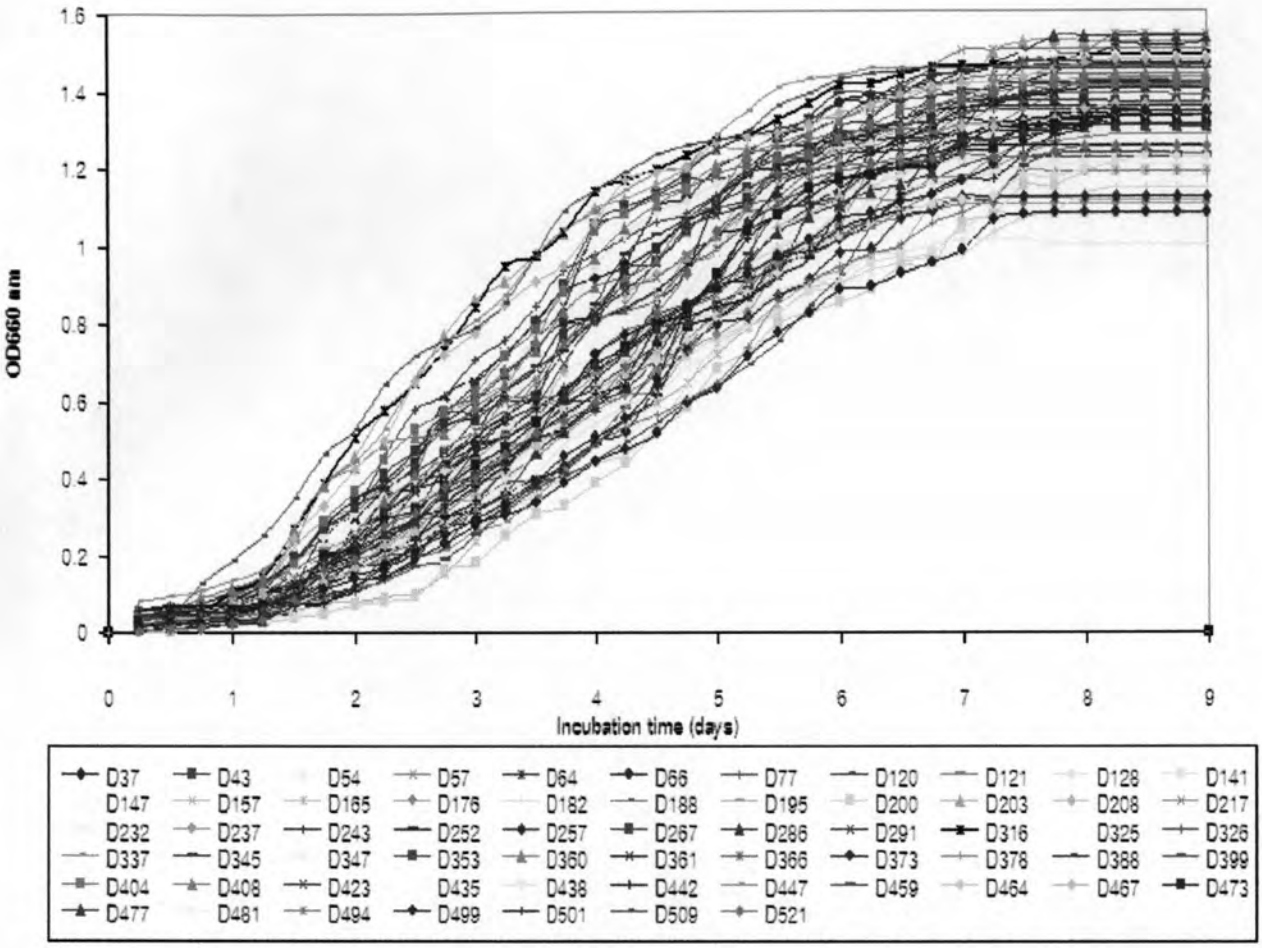

Figure 4.1 (b) Growth curves of 62 bacterial isolates from root nodules of soybean cultivars separately grown in soil samples from Phitsanulok Province. Growth medium was yeast extract mannitol broth. Cell cultures were grown at 200 rpm, 30 °C.

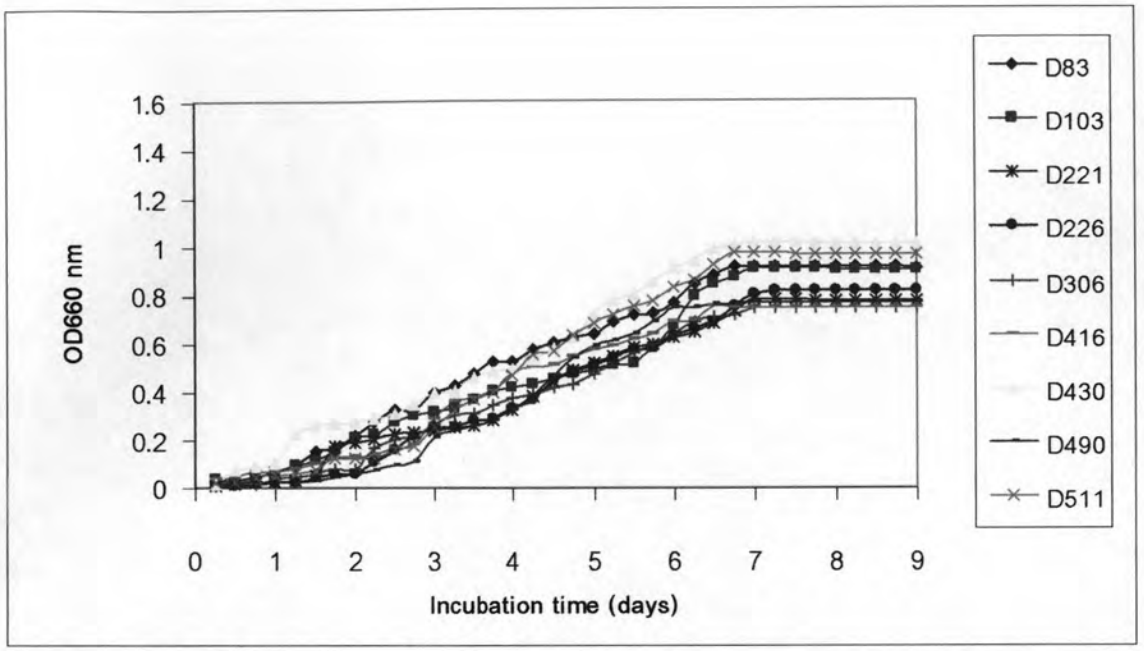

Figure 4.1 (c) Growth curves of 9 bacterial isolates from root nodules of soybean cultivars separately grown in soil samples from Phitsanulok Province. Growth medium was yeast extract mannitol broth. Cell cultures were grown at 200 rpm, 30 °C.

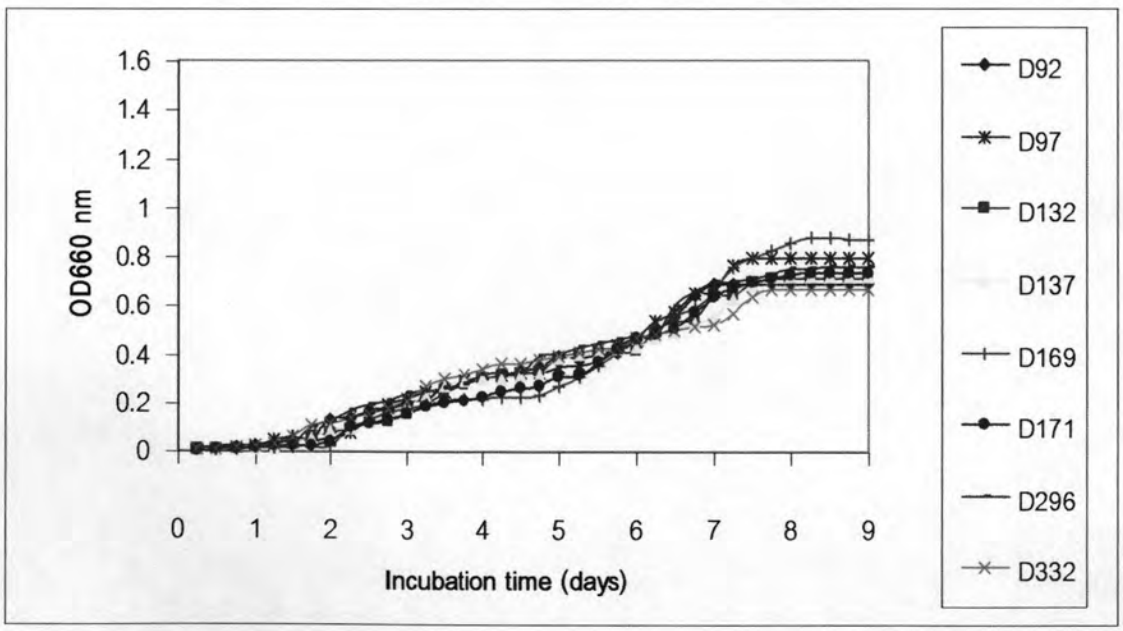

Figure 4.1 (d) Growth curves of 8 bacterial isolates from root nodules of soybean cultivars separately grown in soil samples from 15 subdistricts from Phitsanulok Province. Growth medium was yeast extract mannitol broth. Cell cultures were grown at 200 rpm, 30 °C.

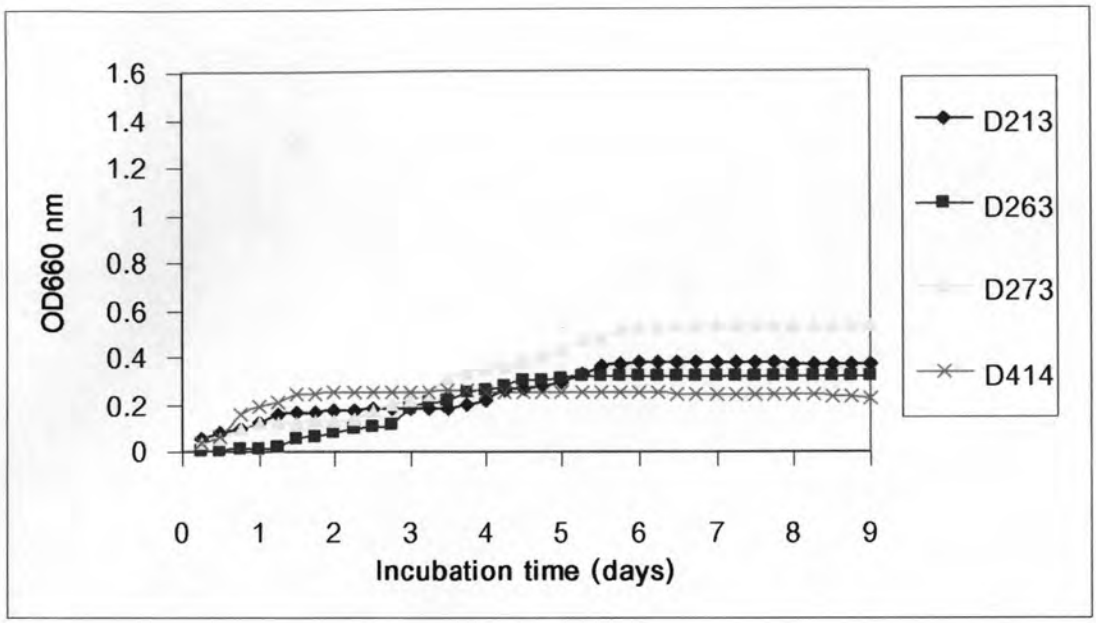

Figure 4.1 (e) Growth curves of 4 bacterial isolates from root nodules of soybean cultivars separately grown in soil samples from Phitsanulok Province. Growth medium was yeast extract mannitol broth. Cell cultures were grown at 200 rpm, 30 °C.

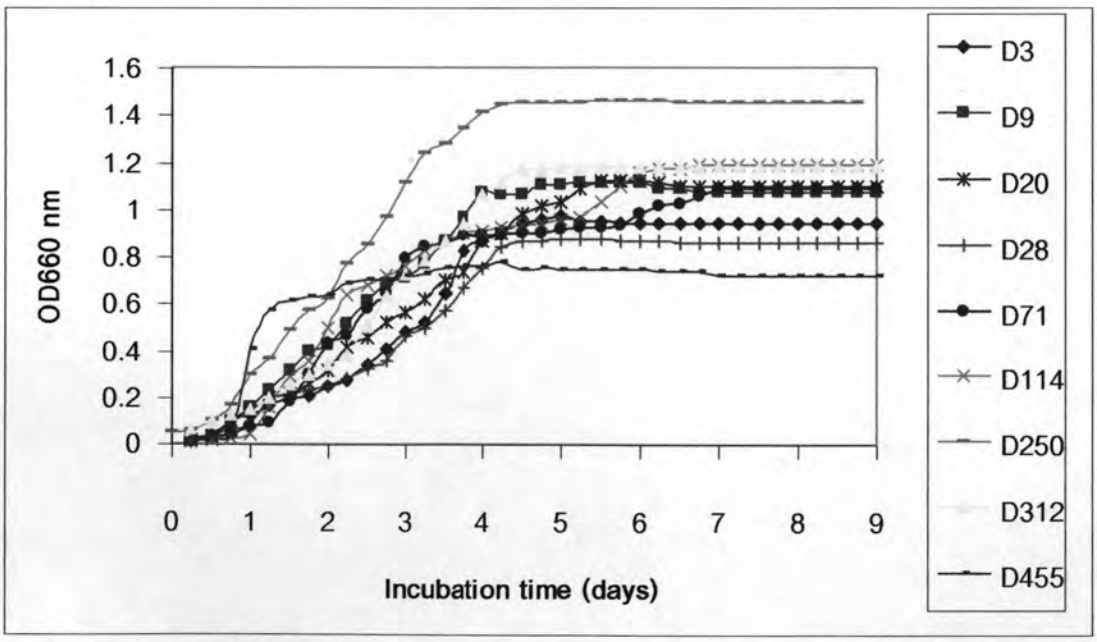

Figure 4.1 (f) Growth curves of 9 bacterial isolates from root nodules of soybean cultivars separately grown in soil samples in Phitsanulok Province. Growth medium was yeast extract mannitol broth. Cell cultures were grown at 200 rpm, 30 °C.

Turbidity profiles of the 105 bacterial isolates from root nodules of soybeans as shown in Figures 4.1 (a) – 4.1 (f) indicated that isolated strains could be grouped into 6 groups with different turbidity profiles. The first group of isolates showed the turbidity increased to an OD_{660} reading of 1.3 in 1 day. This group consisted of 11 isolates as shown in Table 4.4. The second group of isolates increased turbidity to 1.4 in 7.5 days. This group consisted of 62 isolates as shown in Table 4.4. The third group of isolates increased turbidity to OD_{660} of 1.0 in 6.5 days. This group consisted of 9 isolates as shown in Table 4.4. The fourth group of isolates increased turbidity to OD_{660} of 0.8 in 7.25 days. This group consisted of 8 isolates as shown in Table 4.4. The fifth group of isolates increased turbidity to OD_{660} of 0.4 in 5.5 days. This group consisted of 4 isolates as shown in Table 4.4. The sixth group contained isolates with variable times for turbidity to reach stationary phase at OD_{660} 0.6, 1.0, and 1.4 in 2, 4.75, and 4.25 days respectively .The results indicated there were several types of root nodule bacterial isolates with different abilities to increase turbidity to different levels at different times. In this research , the turbidity data were used to group root nodule bacterial isolates into 11 isolates of fast-growing soybean rhizobia (Figure 4.1 (a) , Table 4. 4) and 94 slow- growing soybean rhizobia (Figures 4.1 (b) - 4.1 (f), Table 4.4).

Table 4.4 Grouping of root nodule bacterial isolates according to extent of turbidity at different time periods. Cells were grown in yeast extract mannitol broth at 200 rpm, 30 °C.

Group	Maximum OD_{660} nm at Early stationary phase	Time to Early stationary phase (days)	Isolates code
1	1.3	1	D11, D24, D35, D48, D87, D154, D279, D281, D301, D384, D395 (11 isolates)
2	1.4	7.5	D37, D43, D54, D57, D64, D66, D77, D120, D121, D128, D141, D147, D157, D165, D176, D182, D188, D195, D200, D203, D208, D217, D232, D237, D243, D252, D257, D267, D286, D291, D316, D325, D326, D337, D345, D347, D353, D360, D361, D366, D373, D378, D388, D399, D404, D408, D423, D435, D438, D442,D447, D459, D464, D467, D473,D477, D481, D494, D499, D501, D509, D521 (62 isolates)
3	1.0	6.5	D83, D103, D221, D226. D306, D416, D430, D490, D511 (9 isolates)
4	0.8	7.25	D92, D97, D132, D137, D169, D171, D296 and D332 (8 isolates)
5	0.4	5.5	D213, D263, D273, D414 (4 isolates)
6	0.6, 1.0, 1.4	2, 4.75, 4.25	D3, D9, D20, D28, D71, D114, D250, D312, D455 (9 isolates)

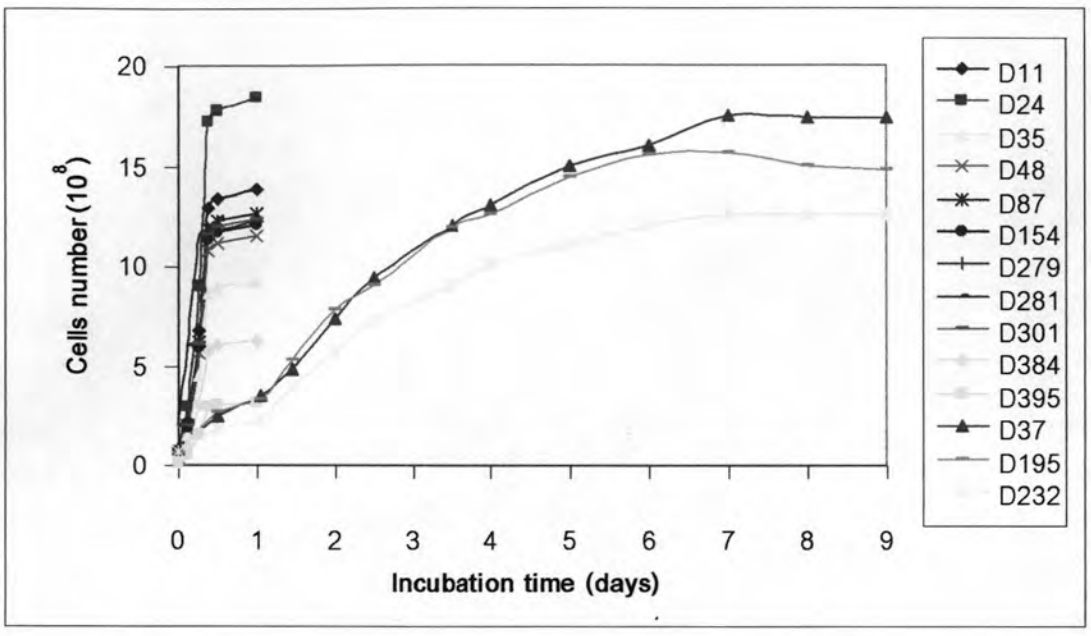

Figure 4.2 Viable plate counts of all the 11 fast-growers and three representatives of slow-growers.

Types of colony morphology

Figure 4.3 showed colony morphology of fast- and slow- growing root nodule bacterial isolates. It was noticed that the 11 fast- growing root nodule bacterial isolates exhibited colonies of different sizes on yeast extract mannitol agar YMA in 7 days at 25 ºC compared to isolates D24, D48, and D154 which were the fastest growing cells that had relatively large colonies (Figure 4.4). The results showed that large colonies did not always indicate that the bacteria were fast- growers. Some fast- growing strains such as D11 and D35 exhibited small colonies when incubated on YMA agar plate during the same incubation time as those fast-growers with large colonies (Figure 4.4). Therefore, a more reliable method, for example, multiplex PCR, is needed to predict the presence of fast- or slow- growing soybean rhizobia.

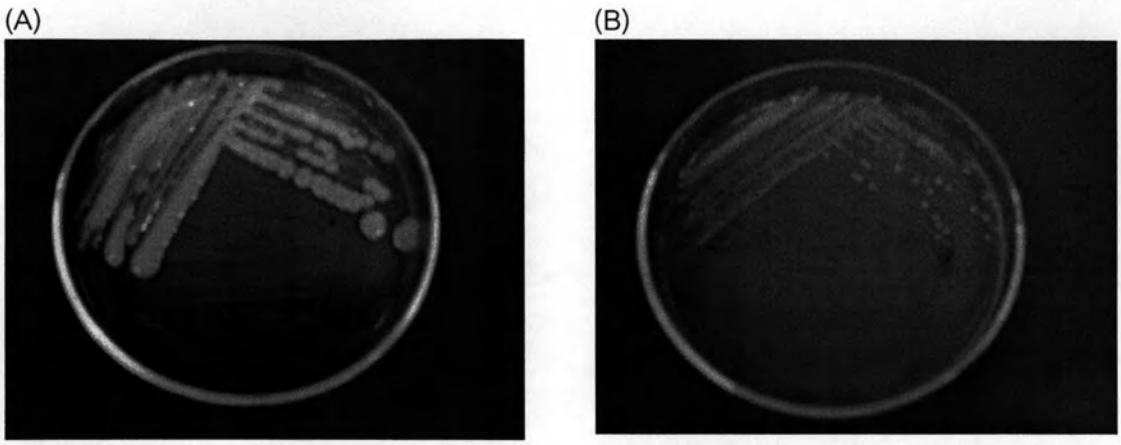

Figure 4.3 Representative colony morphology of (A) fast-growing and (B) slow-growing root nodule bacterial isolates. Each isolate was grown in yeast extract mannitol agar and incubated at 25 °C for 7 days.

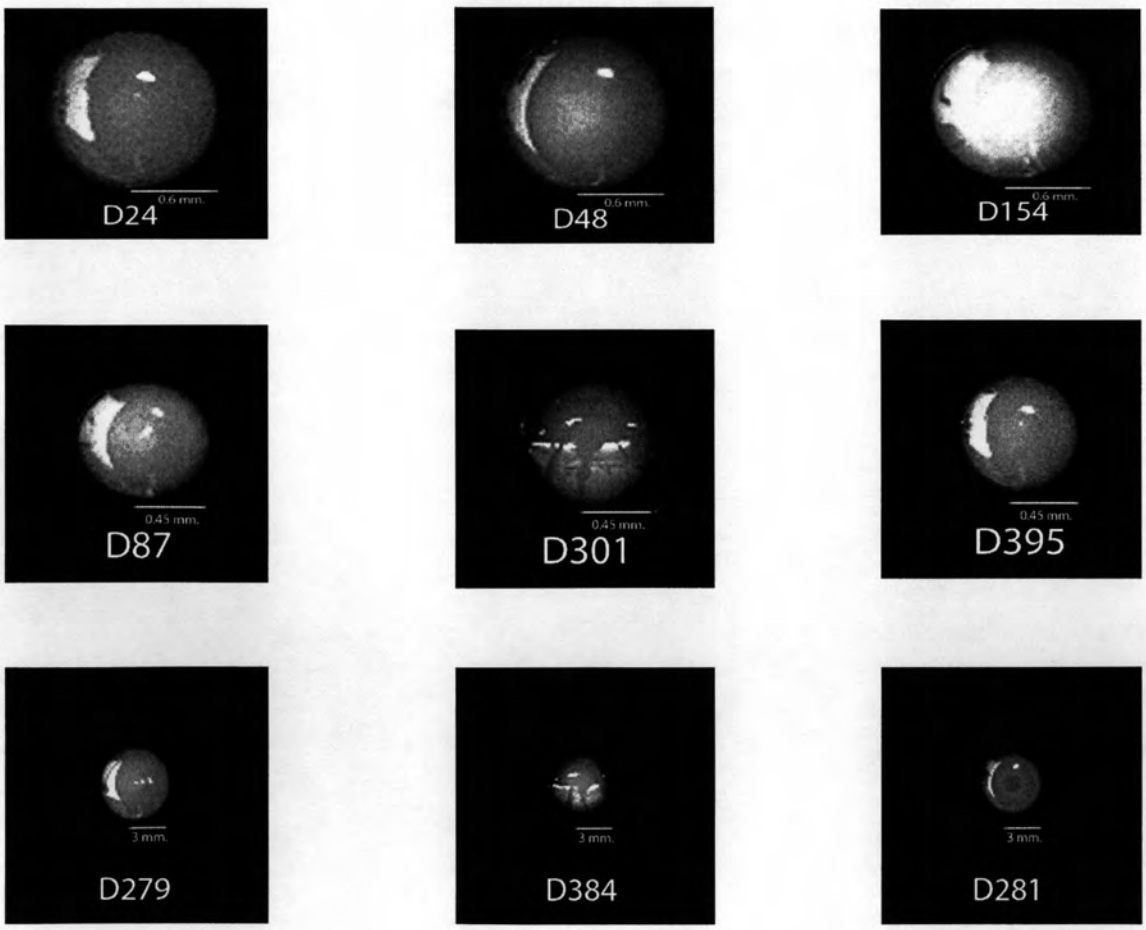

Figure 4.4 Colonies of fast-growing bacteria isolates from root nodules of soybeans. Each isolate was grown in yeast extract mannitol agar and incubated at 25 °C for 4 days.

RAPD-PCR fingerprints of soybean root nodule bacterial isolates

Figures 4.5 to 4.19 showed RAPD-PCR fingerprints of 105 soybean root nodule bacterial isolates when either RPO1 or CRL-7 was used as the primer. Identical RPO1 and CRL-7 fingerprints were used to group different isolates to the same strains as shown in Appendix D and Table 4.5. The results indicated that the 105 isolates could be grouped into 66 strains. Figures 4.20 to 4.25, showed fingerprints of the strains that gave 1, 2, 3, 4, 5 and more than 5 PCR product fragments with RPO1. Table 4.5 indicated that 65 root nodule isolates could be identified as 27 distinct strains. The remaining 40 isolates were found to have distinct RAPD-PCR fingerprints. Therefore, out of the total of 105 bacterial isolates, 67 distinct strains were found. In addition, each strain was found to nodulate more than one local soybean cultivar.

4.2 RAPD-PCR fingerprints of isolates from districts in Phitsanulok Province

Chat Trakarn District

Chart Trakarn subdistrict

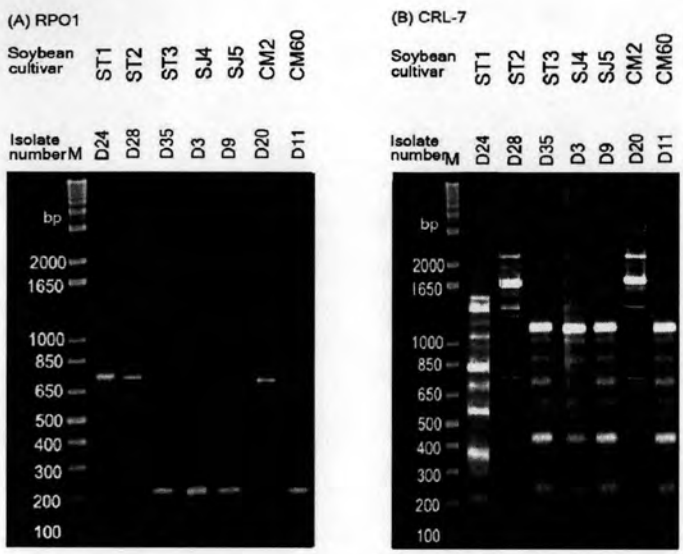

Figure 4.5 RAPD-PCR fingerprints of root nodule bacterial isolates from Chart Trakarn subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Pa Dang subdistrict

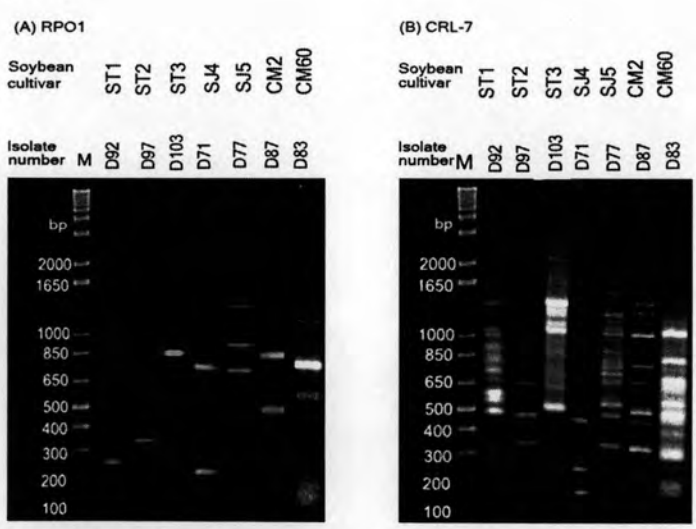

Figure 4.6 RAPD-PCR fingerprints of root nodule bacterial isolates from Pa Dang subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Suan Miang subdistrict

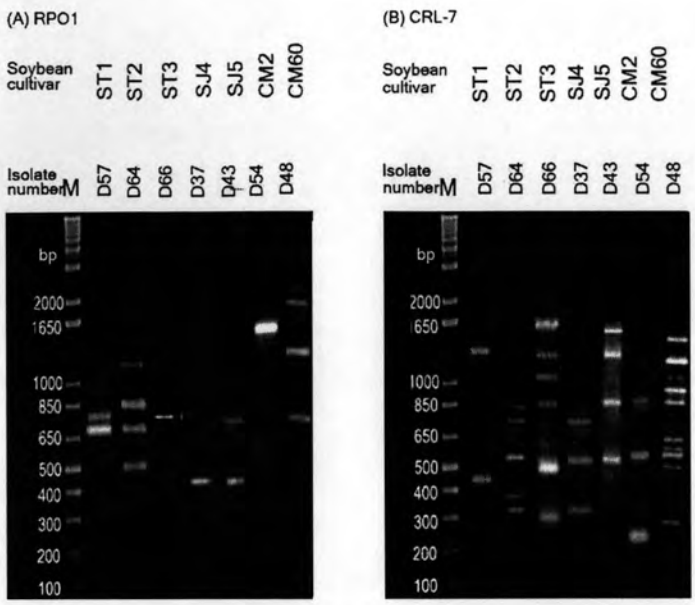

Figure 4.7 RAPD-PCR fingerprints of root nodule bacterial isolates from Suan Miang subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Bang Rakam Districts

Bang Rakam Subdistrict

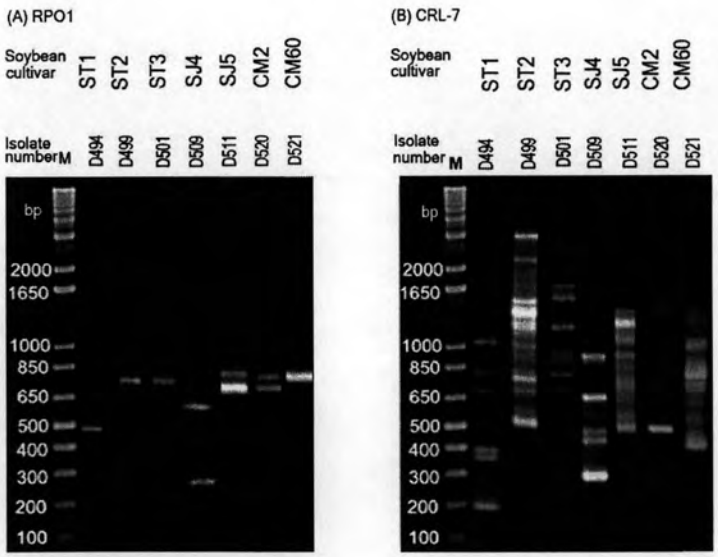

Figure 4.8 RAPD-PCR fingerprints of root nodule bacterial isolates from Bang Rakam subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Pluk Raed subdistrict

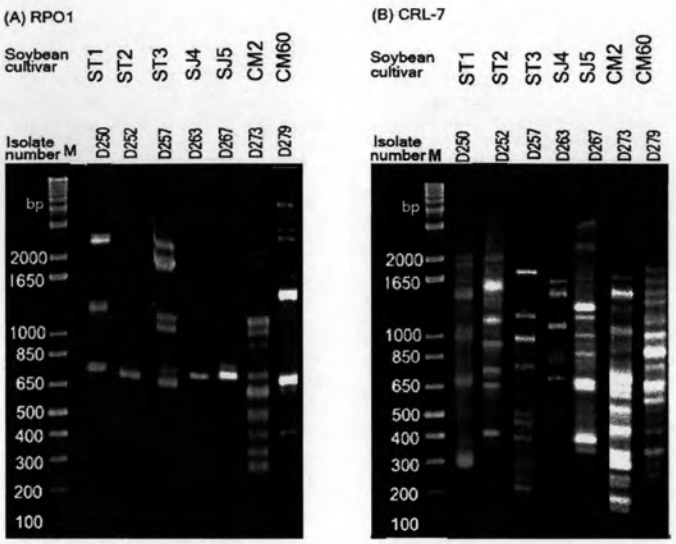

Figure 4.9 RAPD-PCR fingerprints of root nodule bacterial isolates from Pluk Raed subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Pan Sao subdistrict

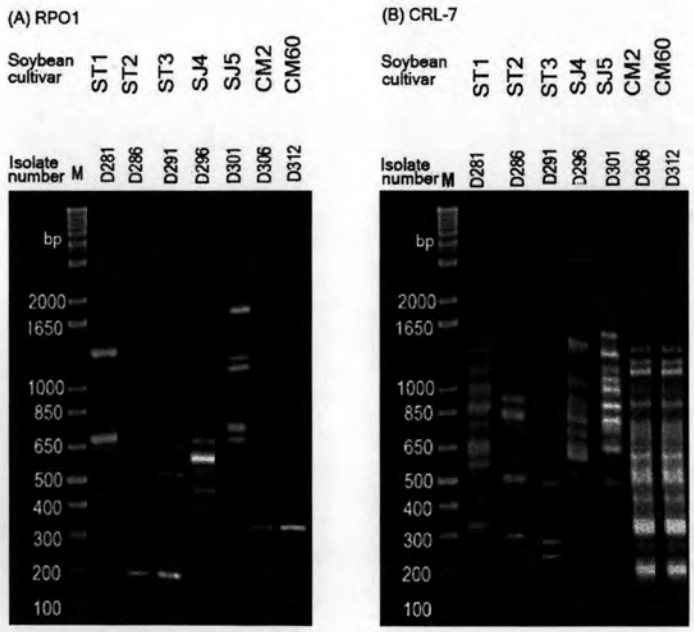

Figure 4.10 RAPD-PCR fingerprints of root nodule bacterial isolates from Pan Sao subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Bung Kok subdistrict

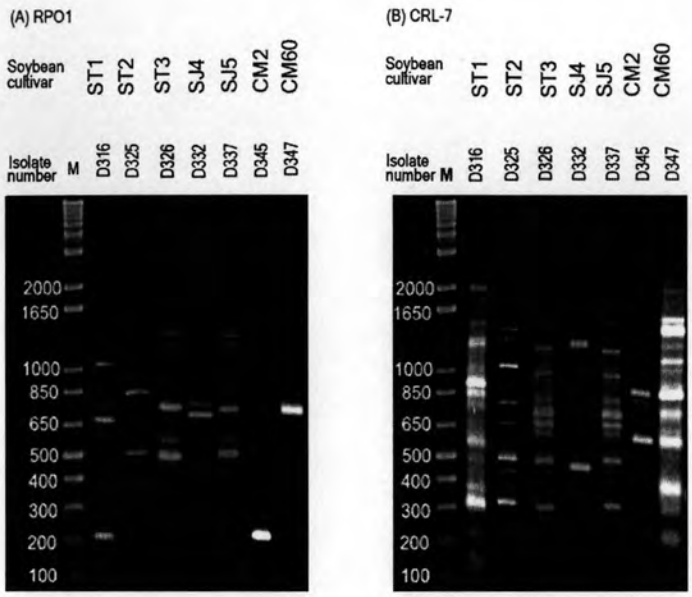

Figure 4.11 RAPD-PCR fingerprints of root nodule bacterial isolates from Bung Kok subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Nong Ku-la subdistrict

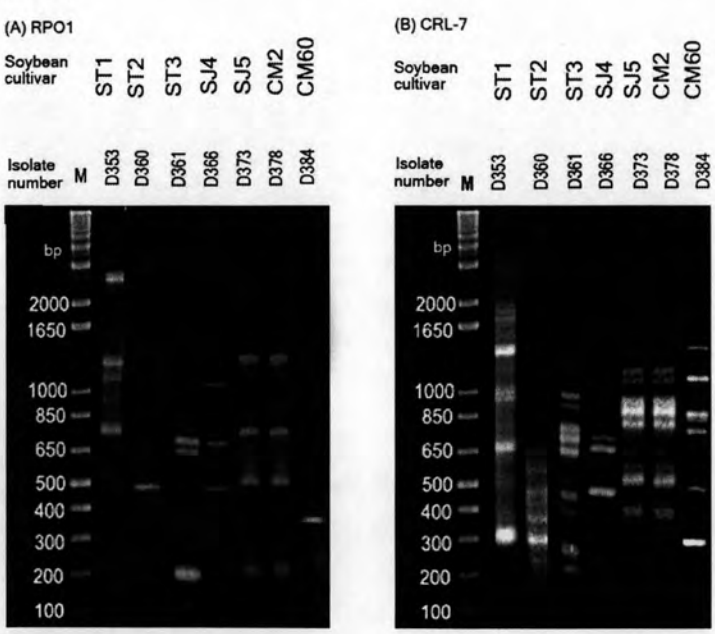

Figure 4.12 RAPD-PCR fingerprints of root nodule bacterial isolates from Nong Ku-la subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Chum Saeng Songkram subdistrict

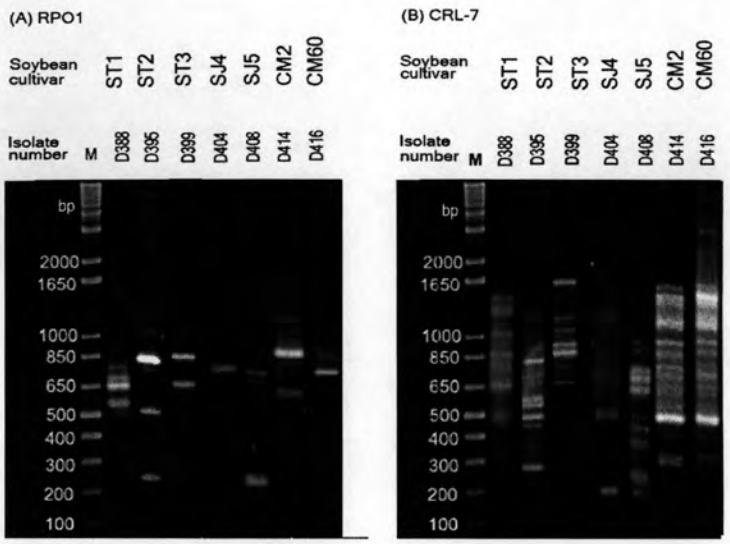

Figure 4.13 RAPD-PCR fingerprints of root nodule bacterial isolates from Chum Saeng Songkram subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Bou Thong subdistrict

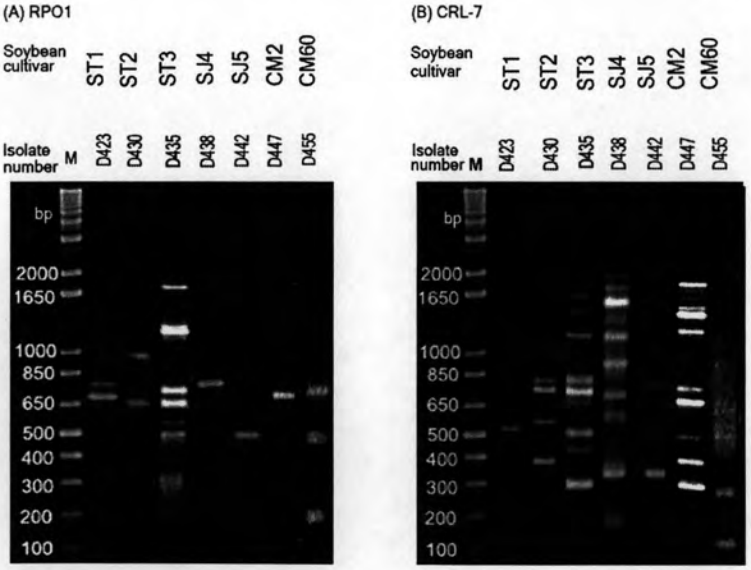

Figure 4.14 RAPD-PCR fingerprints of root nodule bacterial isolates from Bou Thong subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Kui Muang subdistrict

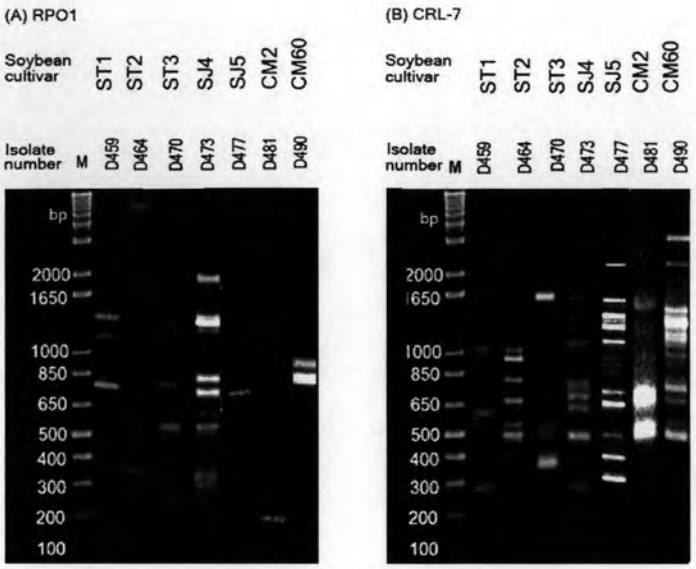

Figure 4.15 RAPD-PCR fingerprints of root nodule bacterial isolates from Kui Muang subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Prom Piram Districts

Wong Kong subdistrict

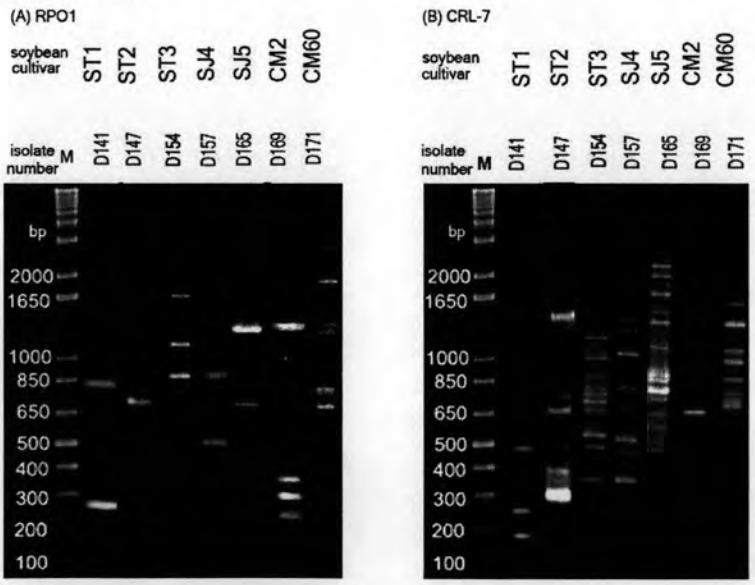

Figure 4.16 RAPD-PCR fingerprints of root nodule bacterial isolates from Wong Kong subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Ta Look Team subdistrict

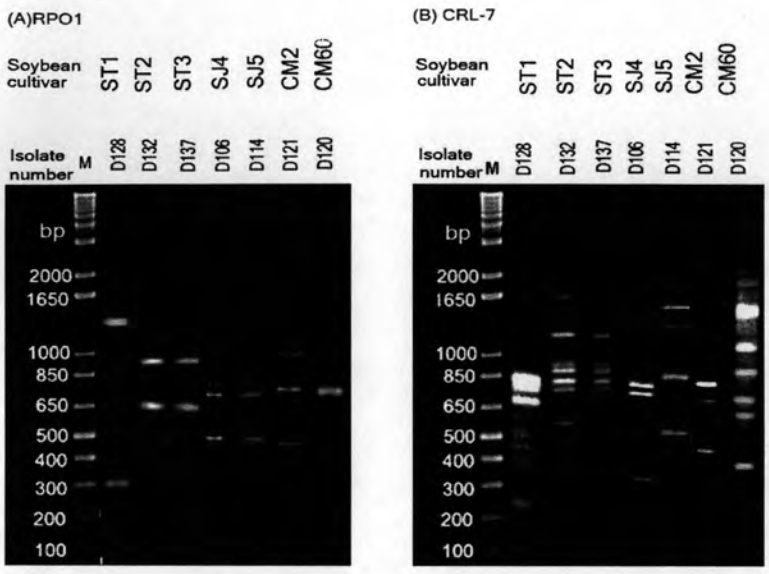

Figure 4.17 RAPD-PCR fingerprints of root nodule bacterial isolates from Ta Look Team subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Wang Won subdistrict

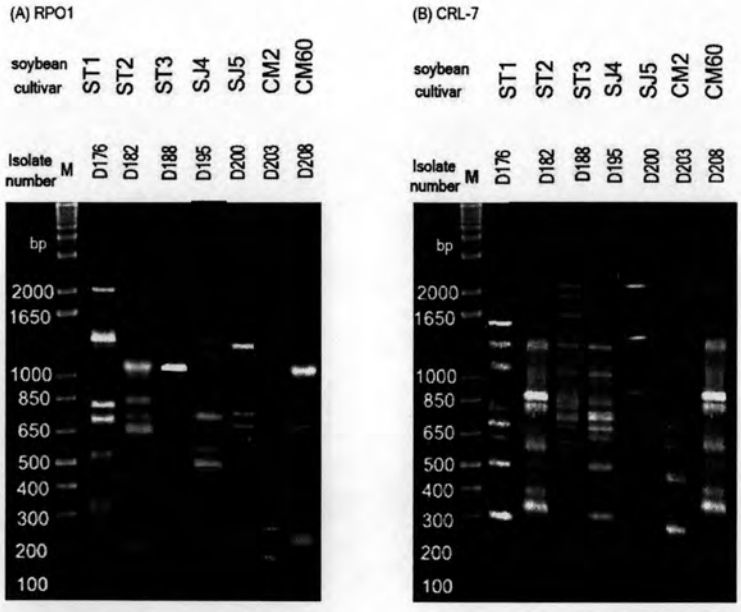

Figure 4.18 RAPD-PCR fingerprints of root nodule bacterial isolates from Wang Won subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

Dong Pa Kam subdistrict

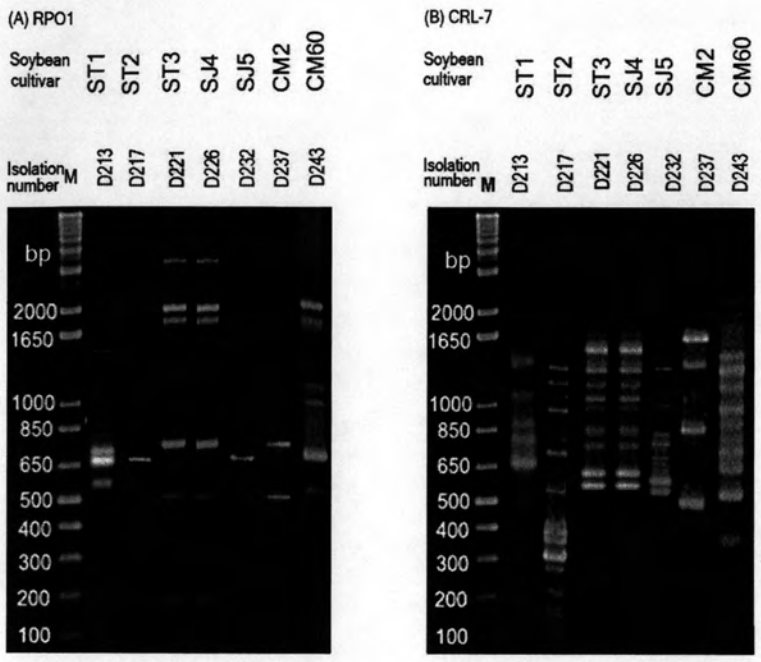

Figure 4.19 RAPD-PCR fingerprints of root nodule bacterial isolates from Dong Pa Kam subdistrict, Phitsanulok Province, when (A) RPO1 or (B) CRL-7 was used as the primer.

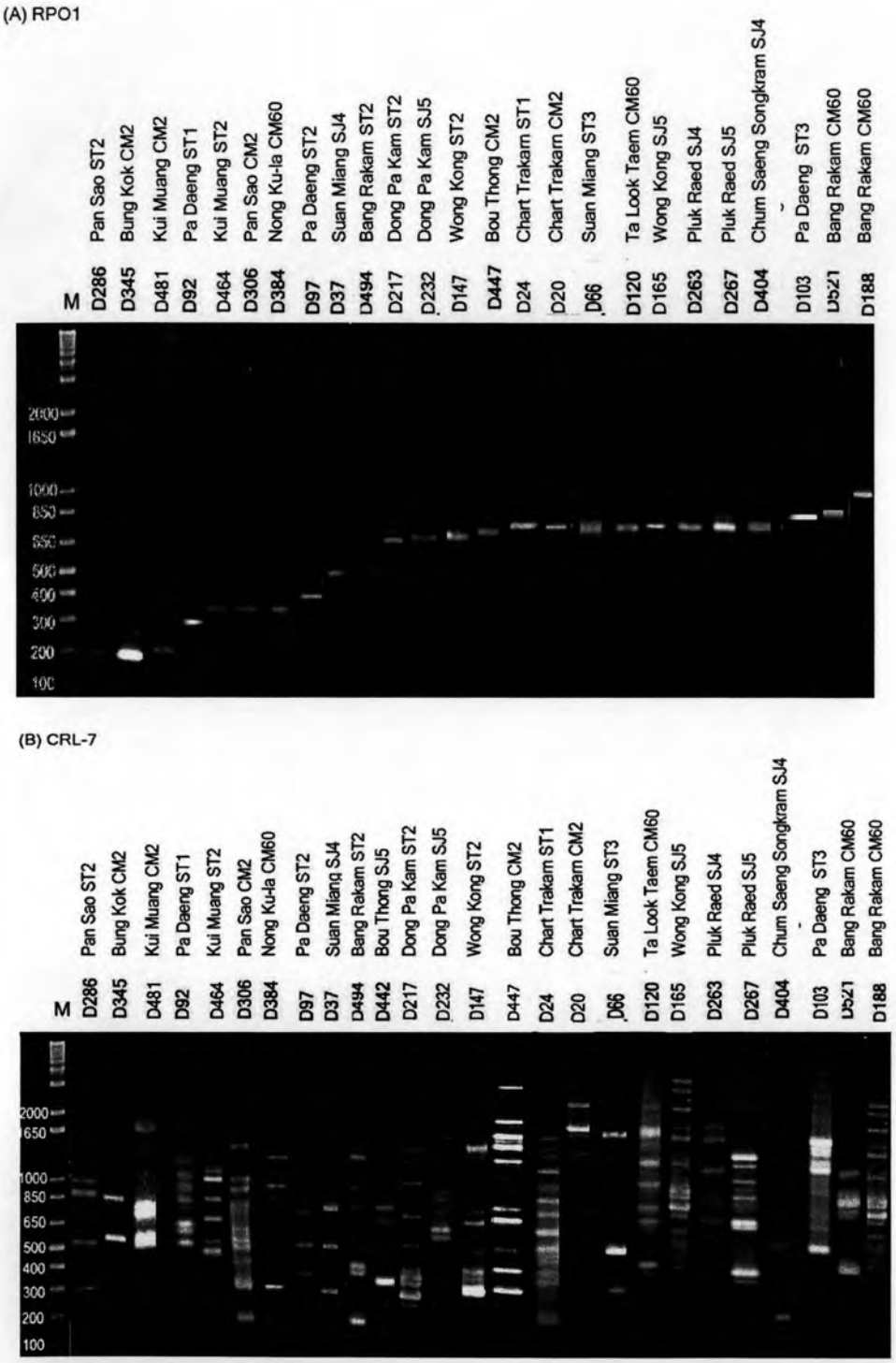

Figure 4.20 RAPD-PCR fingerprints of distinct root nodule bacteria with 1 PRO1-PCR product fragment.

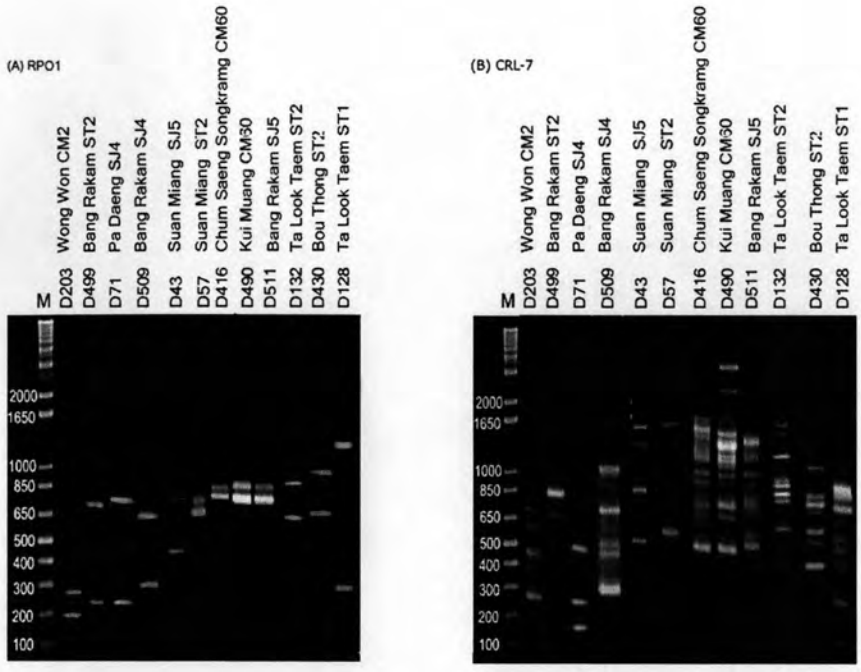

Figure 4.21 RAPD-PCR fingerprints of distinct root nodule bacteria with 2 PRO1-PCR product fragments.

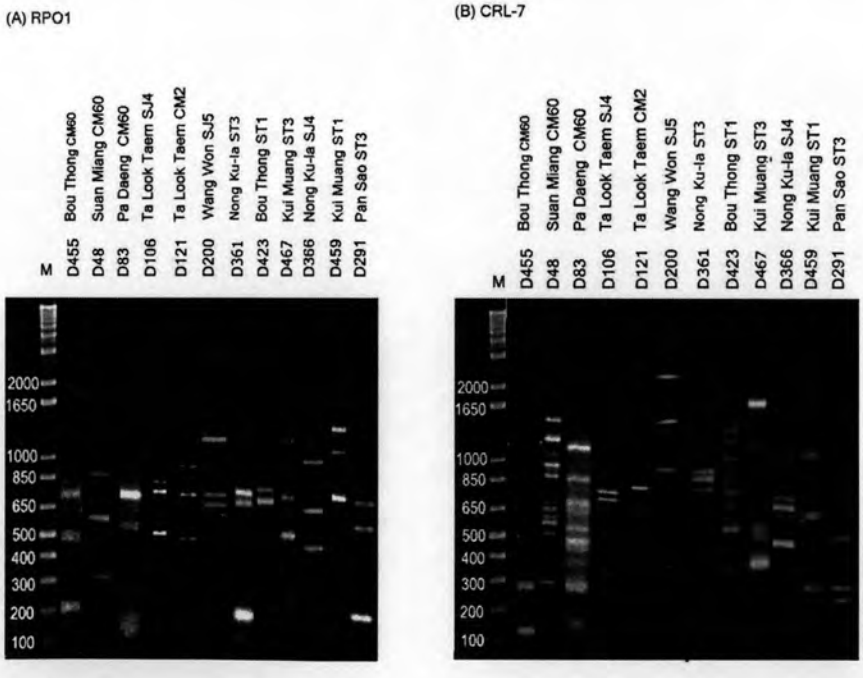

Figure 4.22 RAPD-PCR fingerprints of distinct root nodule bacteria with 3 PRO1-PCR product fragments.

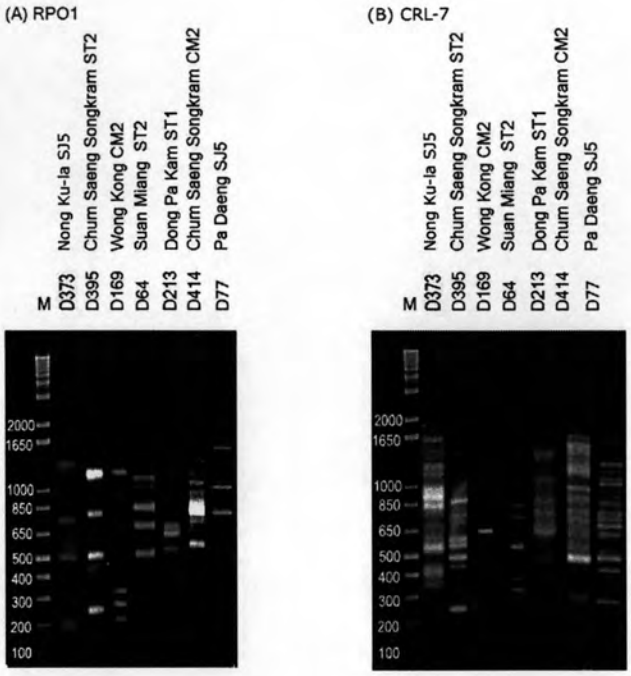

Figure 4.23 RAPD-PCR fingerprints of distinct root nodule bacteria with 4 PRO1-PCR product fragments.

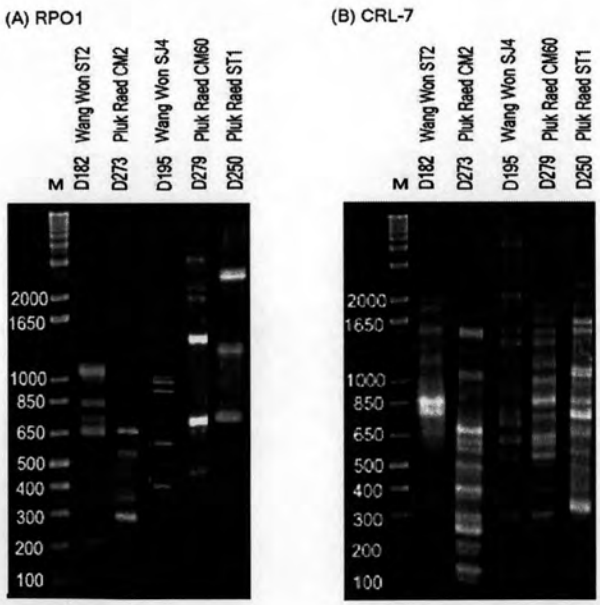

Figure 4.24 RAPD-PCR fingerprints of distinct root nodule bacteria with 5 PRO1-PCR product fragments.

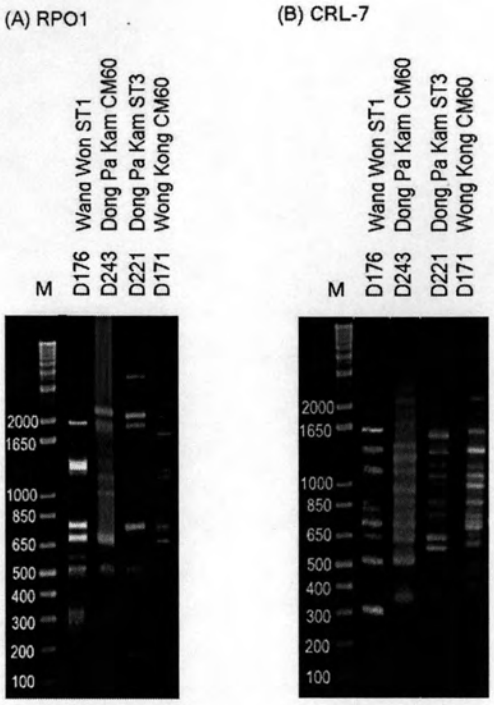

Figure 4.25 RAPD-PCR fingerprints of distinct root nodule bacteria with more than 5 PRO1 PCR product fragments.

Table 4.5 Soybean root nodule bacterial isolates from 15 subdistricts that were the same strains.

Strain	Isolates with identical fingerprints	Sources	
		Soil sample sub-district	Root nodules of soybean cultivars
D3	D3	Chat Trakarn	Sor Jor 4
	D9	Chat Trakarn	Sor Jor 5
	D11*	Chat Trakarn	Chiangmai 60
	D35*	Chat Trakarn	Sor Tor 3
D20	D20	Chat Trakarn	Chiangmai 2
	D28	Chat Trakarn	SorTor 2
D24	D24*	Chat Trakarn	SorTor 1
	D347	Bung Kok	Chiangmai 60
	D438	Bou Thong	SorJor 5
D37	D37	Suan Miang	SorJor 4
	D360	Nong Ku-la	SorTor 2
	D442	Bou Thong	SorJor 5
D43	D43	Suan Miang	SorJor 5
	D114	Ta Look Taem	SorJor 5
	D237	Dong Pa Kam	Chiangmai 2
D57	D57	Suan Miang	SorTor 1
	D332	Bung Kok	SorJor 4
	D520	Bang Rakam	Chiangmai 2
D71	D71	Pa Daeng	SorJor 4
	D141	Wong Kong	SorTor 1
D77	D77	Pa Dang	SorTor 3
	D154*	Wong Kong	SorJor 5

Strain	Isolates with identical fingerprints	Source	
		Soil sample sub-district	Root nodules of soybean cultivars
D87	D87*	Pa Daeng	Chiangmai 2
	D157	Wong Kong	SorJor 4
	D325	Bung Kok	SorTor 2
D120	D120	Ta Look Taem	Chiangmai 60
	D252	Pluk Raed	SorTor 2
D132	D132	Ta Look Taem	SorTor 2
	D137	Ta Look Taem	SorTor 3
	D399	Chum Saeng Songkram	SorTor 2
D171	D171	Wong Kong	Chiangmai 60
	D301*	Pan Sao	SorJor 5
D176	D176	Wang Won	SorTor 1
	D435	Bou Thong	SorTor 3
	D473	Kui Muang	SorJor 4
D182	D182	Wang Won	SorTor 2
	D208	Wang Won	Chiangmai 60
	D316	Bung Kok	SorTor 1
D195	D195	Wang Won	SorJor 4
	D326	Bung Kok	SorTor 3
	D337	Bung Kok	SorJor 5
D213	D213	Dong Pa Kam	SorTor 1
	D296	Pan Sao	SorJor 4
	D388	Chum Saeng Songkram	SorTor 1
D221	D221	Dong Pa Kam	SorTor 3
	D226	Dong Pa Kam	SorTor 3

Strain	Isolates with identical fingerprints	Source	
		Soil sample sub-district	Root nodules of soybean cultivars
D243	D243	Dong Pa Kam	Chiangmai 60
	D257	Pluk Raed	SorTor 3
D250	D250	Pluk Raed	SorTor 1
	D353	Nong Ku-la	SorTor 1
D263	D263	Pluk Raed	SorJor 4
	D501	Bang Rakam	SorTor 3
D279	D279*	Pluk Raed	Chiangmai 60
	D281*	Pan Sao	SorTor 1
D306	D306	Pan Sao	Chiangmai 2
	D312	Pan Sao	Chiangmai 60
D347	D347	Bung Kok	Chiangmai 60
	D438	Bou Thong	SorJor 5
D361	D361	Nong Ku-la	SorTor 3
	D408	Chum Saeng Songkram	SorJor 5
D373	D373	Nong Ku-la	SorJor 5
	D378	Nong Ku-la	Chiangmai 2
D416	D416	Chum Saeng Songkram	Chiangmai 60
	D499	Bang Rakam	SorTor 2
D447	D447	Bou Thong	Chiangmai 2
	D477	Kui Muang	SorJor 5
D490	D490	Kui muang	Chiangmai 60
	D511	Bang Rakam	SorJor5

* = Fast grower

Multiplex PCR

Figure 4.26 showed colony morphology of plant- associated, Gram negative soil bacteria *Agrobacterium tumefacians* TISTR 507 and *Xanthomonas campestris* TISTR 786 which would be used as target DNAs in multiplex PCR. *Proteus vulgaris* which produced extracellular polysaccharides was also used in multiplex PCR to test if the multiplex PCR reactions were specific for fast- and slow- growing soybean rhizobia. Colony morphology as shown in Figure 4.26 indicated that colonies of *Agrobacterium tumefacians* TISTR 507, *Xanthomonas campestris* TISTR 786, and *Proteus vulgaris* on yeast extract mannitol agar plates with 25 $\mu g.ml^{-1}$ Congo red were red, and yellowish – orange due to an ability to absorb Congo- red used in the growth medium. Samasegaran and Hoben (1994) stated that soybean rhizobia did not absorb Congo red in the growth medium while most of other bacteria considered as contaminants did absorb the dye hence contaminant bacterial colonies appeared bright red on YMA with Congo red agar plates. *Xanthomonas campestris* TISTR 786 and *Proteus vulgaris* appeared to produce yellowish- orange carotenoid pigments in YMA medium.

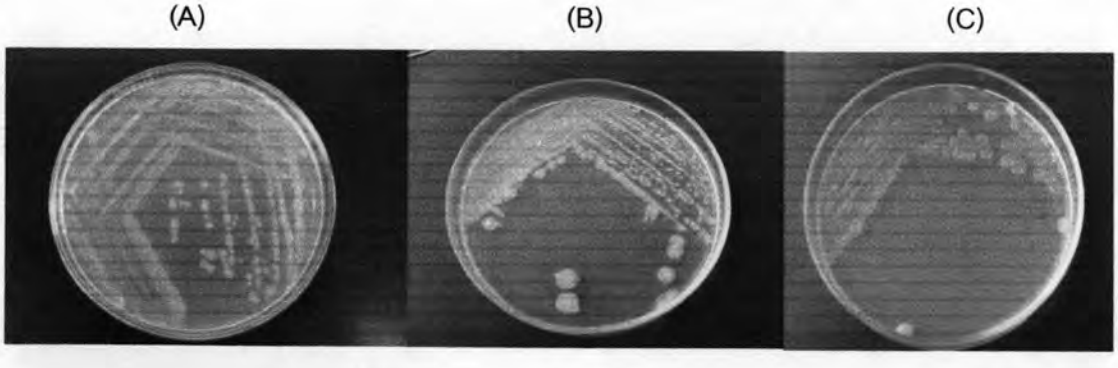

Figure 4.26 Colony morphology of *Agrobacterium tumefacians* TISTR 507, *Xanthomonas campestris* TISTR 786, and *Proteus vulgaris* used in multiplex PCR. Cells were grown on yeast extract mannitol agar with 25 $\mu g.ml^{-1}$ Congo red at 25 ºC for 7 days.

Table 4.6 Properties of forward and reverse primers used in multiplex PCR for detection of fast-growing and slow-growing soybean rhizobia isolated from Chart Trakarn, Bang Rakam and Prom Piram districts, Phitsanulok Province.

Primer	5'-3' sequences	PCR product (bp)	%GC	T_m (°C)
nodD1				
Forward primer	5' AAAATGGCAGCAGYTCGAA 3' (19 bases)	317	42.1	54.3
Reverse primer	5' CAACATCAATCTGAGCCAG 3' (19 bases)			
nodY				
Forward primer	5' TGTACGCGGGTAAACC3' (16 bases)	340	50.0	40.0
Reverse primer	5' AGCGCAACGAGAAGAT3'(16 bases)			

Figure 4.27 showed results of multiplex PCR reactions using forward and reverse primers of *nodD1* as well as forward and reverse primers of *nodY*. When DNAs of slow-growers were used as the template, 340 bp products of *nodY* were detected while no PCR products were obtained when DNAs of fast- growers were used in the multiplex PCR reactions. The results also indicated presence of *nodD1* PCR products of different sizes when DNAs of fast- growers were used in multiplex PCR. Details and interpretation of multiplex PCR results will be elaborated in the Discussion section.

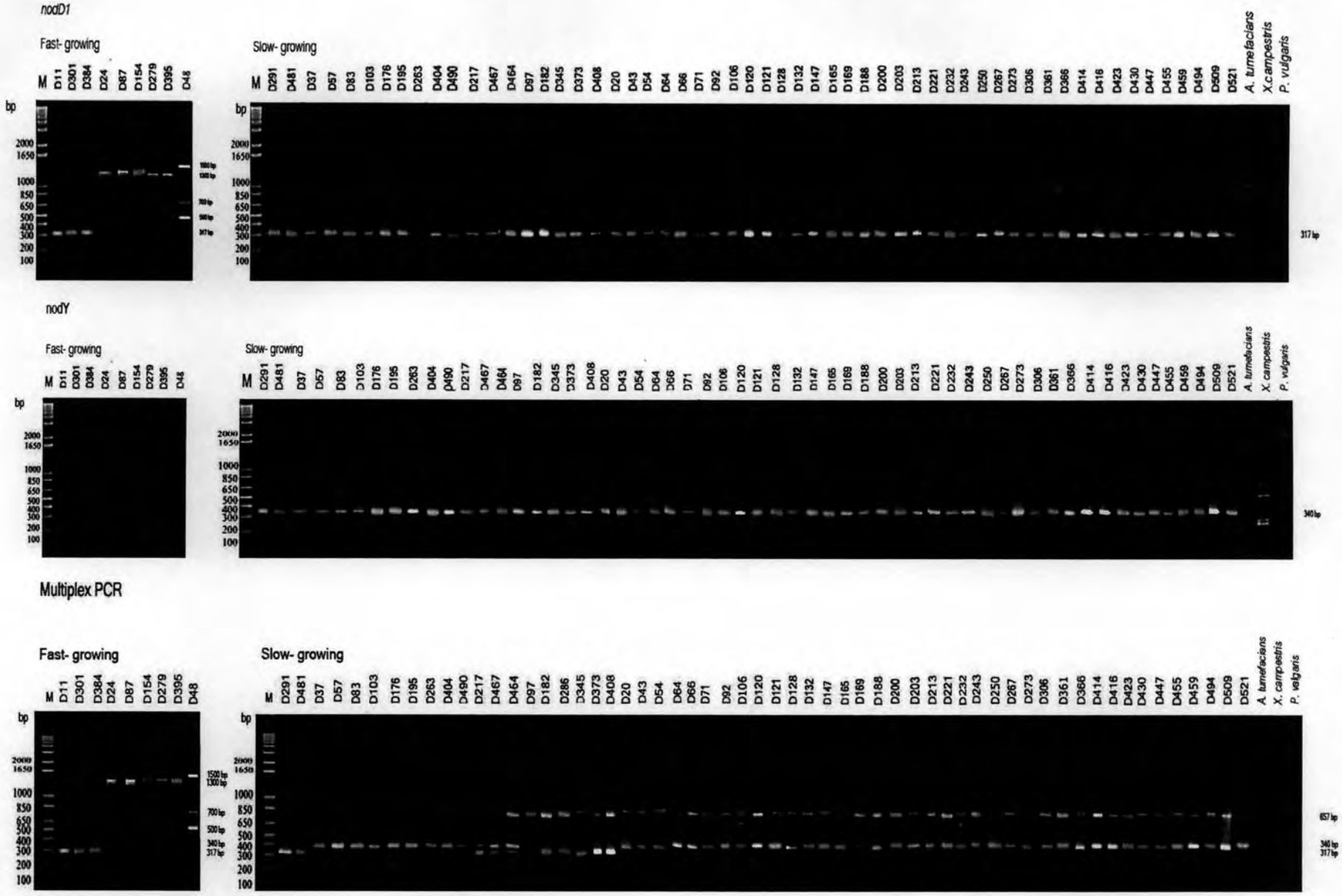

Figure 4.27 PCR products obtained from multiplexPCR reactions.