CHAPTER IV

RESULTS

4.1 Chemical and physical properties of soils from collection sites

Table 4.1 showed chemical and physical properties of soil samples from 16 subdistricts in Phitsanulok province where soil samples were kindly collected in 2006 A.D. by Assistant Professor Dr. Wipa Homhaul, Faculty of Agriculture, Natural Resources and Environment, Naresuan University. Most of the soil samples were acidic with average pHs ranging from 4.30 to 6.67. The organic matter contents were relatively low, ranging from 0.91% in Nong Phra subdistrict to 2.85% in Mathong subdistrict. Moisture contents and water holding capacity were found to be in the range of 0.50% in Ban Dong subdistrict to 12.74% in Sri Phirom subdistrict and 27.29% in Nong Pra subdistrict to 53.44 % in Mathong subdistrict, respectively. The concentrations of metals were as shown in Table 4.2. Na contents were found to range from 70 ppm in Ban Dong subdistrict to 186 ppm in Ban Phrao subdistrict. The results indicated a wide range of Ca contents from 124 ppm in Hua Ro subdistrict to 1948 ppm in Sri Phirom subdistrict. Mg contents were found to have a relatively wide range from 11 ppm in Nong Phra subdistrict to 361 ppm in Mathong subdistrict. A low Fe content of 25.7 was found in Ban Dong subdistrict while high Fe content of 319.2 ppm was found in Mathong subdistrict. Zn, Cu and S contents were low in all soil samples. The results showed soil sample from Ban Dong subdistrict was low in moisture, Na, Fe, and S contents while Mathong soil sample was found to have high water holding capacity and high Mg, Fe, and Zn contents. Sri Phirom soil sample was found to have high moisture, Ca, and Cu contents.

Table 4.1 Chemical and physical properties of soils collected in 2006 A.D. from the collection sites in 16 subdistricts in Phitsanulok province.

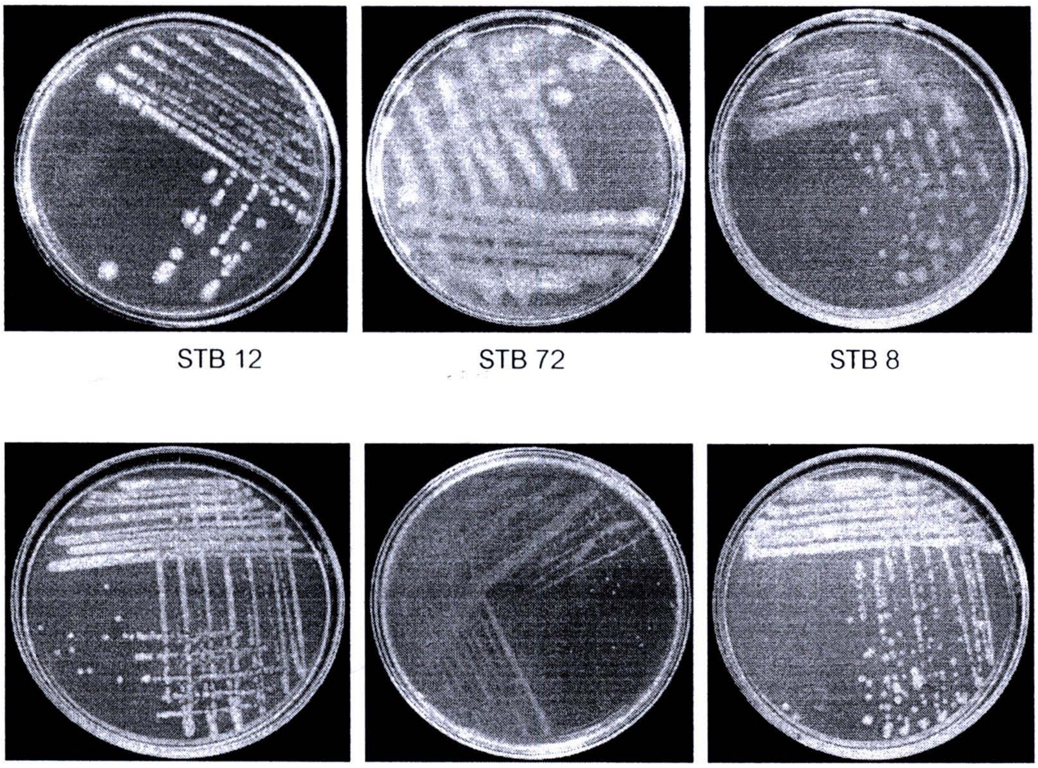
Subdistricts Soil properties	Average soil pH	Organic matter (%)	Available P (ppm)	Available K (ppm)	Water holding capacity (%)	Moisture content (%)
Ban Pa	4.51	1.11	8	90	34.41	1.11
Hua Ro	5.04	1.61	23	62	30.32	0.81
Wat Phrik	6.12	0.94	19	85	33.83	1.42
Wang Ithok	6.30	1.54	27	93	40.11	1.83
Mathong	4.98	2.85	38	115	53.44	2.56
Sri Phirom	4.57	1.98	28	62	41.10	12.74
Tha Chang	6.67	2.38	22	69	42.88	2.15
Ban Dong	5.12	1.11	5	49	27.91	0.50
Ban Phrao	4.68	2.64	32	83	43.56	2.99
Nakhon Chum	4.30	2.38	20	48	32.14	1.21
Nong Kathao	5.52	1.80	26	75	35.77	2.04
Chaiyanam	5.51	2.48	17	185	40.99	2.35
Kaeng Sopha	5.50	1.88	33	75	34.35	1.73
Mae Raka	4.60	2.18	7	105	40.56	1.73
Nong Phra	4.92	0.91	9	20	27.29	0.60
Tha Muen Ram	4.70	1.98	30	144	35.31	1.52

Table 4.2 Concentrations of metals in soil samples collected in 2006 A.D. from the collection sites in 16 subdistricts in Phitsanulok province (units in ppm).

Subdistricts Concentrations of metals	Na	Ca	Mg	Fe	Mn	Zn	Cu	Cl$^-$	S
Ban Pa	106	315	81	127.0	35.3	1.35	1.04	26.6	13
Hua Ro	82	124	48	147.7	46.1	0.73	0.35	53.3	2
Wat Phrik	96	1072	278	22.7	44.4	0.75	1.18	53.3	36
Wang Ithok	94	1164	255	85.5	61.3	1.23	2.04	26.6	16
Mathong	168	1490	361	319.2	118.0	2.83	4.16	53.3	44
Sri Phirom	151	1948	306	287.0	85.1	2.17	2.98	53.3	24
Tha Chang	104	131	122	77.8	74.5	1.80	2.75	53.3	11
Ban Dong	70	2156	132	25.7	30.9	0.39	0.38	26.6	1
Ban Phrao	186	224	43	237.5	141.8	2.39	1.65	80.0	20
Nakhon Chum	81	1240	156	199.3	36.6	2.75	0.65	53.3	3
Nong Ka Thao	157	1321	124	174.4	122.0	1.68	1.57	79.9	70
Chaiyanam	145	831	127	80.0	121.6	2.05	1.61	53.3	18
Kang Sopha	85	1130	131	83.8	123.2	2.20	1.39	79.9	2
Mae Raka	183	406	150	191.8	170.1	1.94	1.34	26.6	10
Nong Phra	82	342	11	57.1	26.7	0.52	0.39	26.6	1
Tha Muen Ram	132	104	83	227.8	115.0	1.78	1.23	53.3	21

4.2 Isolation and characterization of bacteria from root nodules of soybeans

Table 4.3 showed 340 bacterial isolates obtained by the host trapping method using 5 soybean cultivars. The isolates were categorized into fast- growers based on the visibility of colonies within 24 h growth on yeast extract mannitol agar (YMA) containing 25 ug.ml^{-1} congo red. Those isolates with colonies visible on YMA with congo red plates after at least 5-day incubation were recoded as slow-growers. Appendix C showed colony morphology of all 340 isolates. Figure 4.1 showed colony morphology of representative isolates.



STB 12 STB 72 STB 8

STB96 STB336 STB264

Figure 4.1 Colony morphology of some representative fast- (STB 12, STB 72) and slow-growing bacteria isolated from root nodules of soybeans (STB 8, STB 96, STB336, and STB 264).

The results as shown in Figure 4.1 showed there were two and four types of colonies for fast- and slow-growing bacteria isolated from root nodules of soybeans respectively. After 24 h growth on YMA plates, the first type of colonies of fast-growers as represented by STB12 was opaque and regular while the second type as represented by STB72 was relatively large, round (diameter up to 0.5 mm), and slimy. Colonies of the isolated slow-growing bacteria on YMA plates after 5-day incubation were small with up to 0.1 mm in diameter. The first type of colonies as represented by STB8 was irregular and slimy . The second type (STB96) was small, round and pearly while the third type was very small with diameter less than 0.1 mm (STB336) and the fourth type was round and shiny as represented by STB264 in Figure 4.1. Table 4.3 showed codes of bacteria isolated by the host trapping method from root nodules of 5 soybean cultivars. The Table indicated the nature of fast- and slow-growing.

Table 4.3 Bacteria isolated from root nodules of 5 soybean cultivars grown in soils from 16 subdistricts in Phitsanulok province.

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
Ban Pa	ST 1	STB 1	S
		STB 2	F
		STB 3	S
		STB 4	S
	ST 2	STB 5	S
		STB 6	S
	SJ 5	STB 7	F
		STB 8	S
		STB 9	S
		STB 10	S
	CM 2	STB 11	F
		STB 12	F
		STB13	F
	CM 60	STB 14	S
		STB 15	S
		STB 16	S
		STB 17	F
		STB 18	F
		STB 19	F
		STB 20	S
Hua Ro	ST 1	STB 21	S
		STB 22	S
		STB 23	F
	ST 2	STB 24	F
		STB 25	F
		STB 26	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
		STB 27	F
		STB 28	S
		STB 29	S
	SJ 5	STB 30	S
		STB 31	F
		STB 32	F
		STB 33	F
		STB 34	F
	CM 2	STB 35	F
		STB 36	F
		STB 37	F
		STB 38	S
		STB 39	F
		STB40	F
	CM 60	STB 41	S
		STB 42	F
		STB 43	F
		STB 44	F
Wat Phrik	ST 1	STB 45	F
		STB 46	S
		STB 47	F
		STB 48	F
		STB 49	F
	ST 2	STB 50	S
		STB 51	S
	ST 2	STB 52	S
		STB 53	S
		STB 54	S
	SJ 5	STB 55	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	SJ5	STB 56	F
		STB 57	S
		STB 58	S
		STB 59	S
	CM 2	STB 60	S
		STB 61	S
		STB 62	S
	CM 60	STB 63	S
		STB 64	S
		STB 65	F
		STB 66	F
Wang Ithok	ST 1	STB 67	S
		STB 68	S
		STB 69	S
		STB 70	S
		STB 71	S
	ST 2	STB 72	F
		STB 73	F
		STB 74	F
	SJ 5	STB 75	F
		STB 76	F
		STB 77	F
		STB 78	F
		STB 79	F
	CM 2	STB 80	F
		STB 81	F
		STB 82	F
		STB 83	F
		STB 84	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM2	STB 85	F
		STB 86	F
	CM 60	STB 87	F
		STB 88	F
		STB 89	S
		STB 90	F
Mathong	ST 1	STB 91	F
		STB 92	F
		STB 93	F
		STB 94	F
	ST 2	STB 95	S
		STB 96	S
		STB 97	F
		STB 98	S
		STB 99	S
	SJ 5	STB 100	S
		STB 101	F
		STB 102	F
		STB 103	F
		STB 104	S
		STB 105	S
	CM 2	STB 106	S
		STB 107	S
		STB 108	S
		STB 109	S
	CM 60	STB 110	F
		STB 111	F
		STB 112	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	ST 1	STB 113	S
		STB 114	S
		STB 115	S
	ST 2	STB 116	S
		STB 117	S
		STB 118	S
		STB 119	S
Sri Phirom	SJ 5	STB 120	S
		STB 121	F
		STB 122	F
		STB 123	F
	CM 2	STB 124	S
		STB 125	F
		STB 126	S
		STB 127	S
		STB 128	F
		STB 129	F
		STB 130	F
	CM 60	STB 131	S
		STB 132	S
		STB 133	S
		STB 134	S
Tha Chang	ST 1	STB 135	S
		STB 136	S
		STB 137	S
		STB 138	S
	ST 2	STB 139	S
		STB 140	S
		STB 141	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	ST 2	STB 142	S
		STB 143	F
	SJ 5	STB 144	F
		STB 145	F
		STB 146	F
	CM 2	STB 147	S
		STB 148	F
		STB 149	F
	CM 60	STB 150	S
		STB 151	S
		STB 152	F
		STB 153	F
Ban Dong	ST 1	STB 154	S
		STB 155	F
		STB 156	S
	ST 2	STB 157	S
		STB 158	S
		STB 159	F
		STB 160	F
		STB 161	F
		STB 162	S
		STB 163	S
		STB 164	F
	SJ 5	STB 165	F
		STB 166	F
		STB 167	F
	CM 2	STB 168	S
		STB 169	S
		STB 170	F

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM2	STB 171	S
		STB 172	F
		STB 173	S
		STB 174	F
		STB 175	F
		STB 176	S
		STB 177	S
	CM 60	STB 178	S
		STB 179	S
		STB 180	S
		STB 181	S
		STB 182	S
		STB 183	S
		STB 184	S
		STB 185	S
Ban Phrao	ST 1	STB 186	S
		STB 187	S
	ST 2	STB 188	S
		STB 189	S
		STB 190	S
		STB 191	F
		STB 192	F
		STB 195	F
		STB 196	F
	CM 2	STB 197	F
		STB 198	S
		STB 199	F
		STB 200	S
	CM 60	STB 201	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM 60	STB 202	S
		STB203	S
Nakhon Chum	ST 1	STB 204	S
		STB 205	F
		STB 206	S
		STB 207	F
		STB 208	F
	ST 2	STB 209	S
		STB 210	F
		STB 211	F
		STB 212	F
		STB 213	S
		STB 214	S
	CM 2	STB 219	F
		STB 220	S
	CM 60	STB 221	S
		STB 222	S
		STB 223	S
		STB 224	S
		STB 225	F
Nong Ka Thao	ST 1	STB 226	S
		STB 227	S
		STB 228	S
		STB 229	F
		STB 230	F
	ST 2	STB 231	S
		STB 232	S
		STB 233	S
		STB 234	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM 2	STB 241	S
		STB 242	S
		STB 243	S
		STB 244	F
		STB 245	S
	CM 60	STB 246	S
		STB 247	F
		STB 248	S
		STB 249	F
		STB 250	S
Chaiyanam	ST 1	STB 251	S
		STB 252	S
	ST 2	STB 253	S
		STB 254	S
	SJ 5	STB 324	S
		STB 325	S
		STB 326	S
	CM 2	STB 255	S
		STB 256	S
		STB 257	F
		STB 258	F
	CM 60	STB 259	S
		STB 260	S
		STB 261	S
Kang Sopha	ST 1	STB 262	F
		STB 263	F
		STB 264	S
		STB 265	S
	ST 2	STB 266	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	ST 2	STB 267	F
		STB 268	S
		STB 269	S
	SJ 5	STB 270	S
		STB 271	S
		STB 272	S
	CM 2	STB 273	S
		STB 274	S
		STB 275	S
	CM 60	STB 276	S
		STB 277	F
		STB 278	F
		STB 279	F
		STB 280	S
Mae Raka	ST 1	STB 281	S
		STB 282	S
		STB 283	F
	ST 2	STB 284	S
		STB 285	S
		STB 286	S
		STB 287	S
		STB 288	S
	SJ 5	STB 289	F
		STB 290	F
		STB 291	F
	CM 2	STB 327	S
		STB 328	S
		STB 329	S
	CM 60	STB 292	S

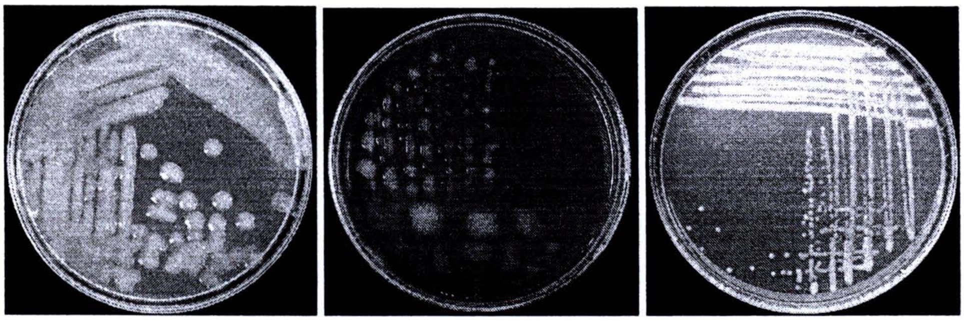
Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	CM 60	STB 293	F
		STB 294	S
Nong Phra	ST 1	STB 295	S
		STB 296	S
		STB 297	F
		STB 298	S
		STB 299	S
	ST 2	STB 330	S
		STB 331	S
		STB 332	S
	SJ 5	STB 300	S
		STB 301	F
		STB 302	S
		STB 303	F
		STB 304	F
	CM 2	STB 333	S
		STB 334	S
		STB 335	S
	CM 60	STB 305	F
		STB 306	S
		STB 307	F
		STB 308	S
		STB 309	F
Tha Muen Ram	ST 1	STB 310	S
		STB 311	F
		STB 312	F
		STB 313	F
		STB 314	F
	ST 2	STB 315	S

Subdistricts	Cultivars of soybean host	Code of bacterial isolates	Fast (F) or Slow (S) growers
	SJ 5	STB 336	S
		STB 337	S
		STB 338	S
	CM 2	STB 339	S
		STB 340	S
	CM 60	STB 320	S
		STB 321	S
		STB 322	F
		STB 323	S

The results as shown in Table 4.3 indicated 138 and 202 isolates of fast- and slow-growing bacteria were obtained respectively. It was noted that in Wang Ithok subdistrict all bacteria isolated from root nodules of soybean cultivars ST2, SJ5, and CM2 were fast-growers. Only fast-growing bacteria were also isolated from root nodules of soybean cultivar SJ5 when soybeans were grown in soils from Tha Chang, Ban Dong, Ban Phrao, and Mae Raka subdistricts. Isolation of bacteria from root nodules of soybean cultivar CM2 grown in soil from Ban Pa also showed only fast-growing bacteria were obtained. Other soil-soybean cultivar combinations where only fast-growing bacteria were isolated were Mathong-ST1 and Mathong-CM60. Physical characteristics of soil samples from Wang Ithok and Tha Chang subdistricts, as shown in Tables 4.1 and 4.2, showed there were no distinct soil properties in these two subdistricts when compared with physical properties of soil samples collected from other subdistricts except that the two soil samples from Wang Ithok and Tha Chang were least acidic with pHs 6.30 and 6.67. It is not known if the relativey high soil pHs were conducive to more fast-growing bacteria. In terms of the soybean cultivars used as the host traps, in order to attract more fast-growing bacteria to the root nodules, the attractant flavonoid signal molecules secreted by roots of soybean cultivar SJ5 might be qualitatively and/or quantitatively different from those secreted by roots of other soybean cultivars. There have been reports that different soybean cultivars secrete different kinds and different quantities of the attractant flavonoid molecules. Since RAPD-PCR fingerprinting of all the 340 isolates to determine which isolates were the same strains was time-consuming, it was decided that, instead of waiting for the results of the determination of which isolates belonged to the same strains, determination of bromthymol blue reactions and authentication of all the fast- and slow-growing isolates would be carried out for all isolates.

4.3 Bromthymol blue reactions

The results of Bromthymol blue (BTB) reactions of some representative fast- and slow-growing isolates were shown in Figure 4.2. The results showed that all the fast-growing isolates secreted acidic product(s) to turn the dye to yellow color. There were two types of BTB reactions for the slow-growing isolates with the unexpected results for the slow-growing isolates. Somasegaran and Hoben (1994) stated that fast- growing soybean rhizobia secreted acidic product(s) which turned the color of the indicator bromthymol blue into yellow while slow- growing soybean rhizobia secreted alkali product(s) which renderedd the bromthymol blue indicator blue color. The results obtained from this experiment indicated that, based on the bromthymol blue reactions, the slow-growing isolates could be divided into two groups with group 1 containing the isolates which secreted alkali product(s) throughout the 10-day incubation period while group 2 consisted of the slow-growing isolates which secreted alkali products after 5-day incubation and acidic product(s) was secreted after 10-day incubation. Figure 4.2 showed bromthymol blue reaction of some representative of fast- and slow-growing isolates.

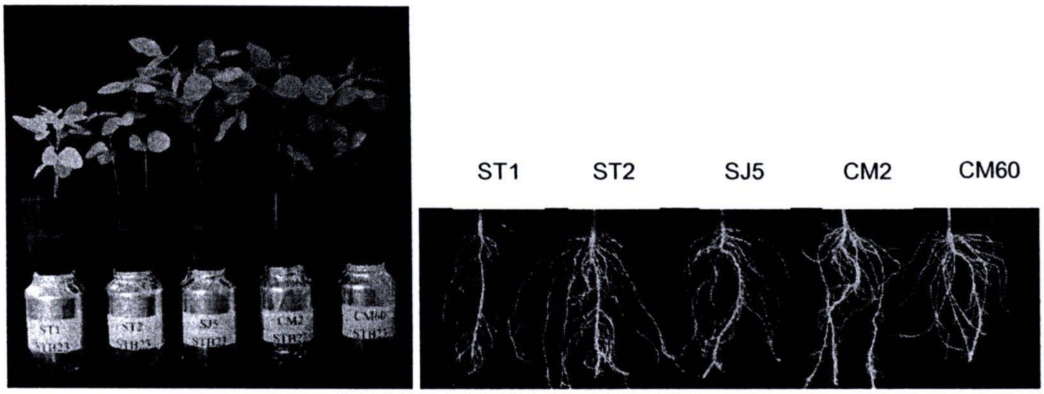


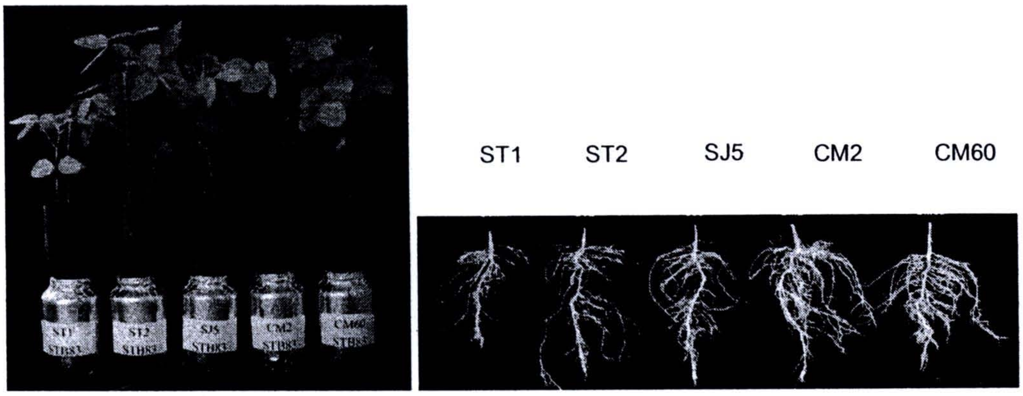
STB 97 STB 8 STB 30

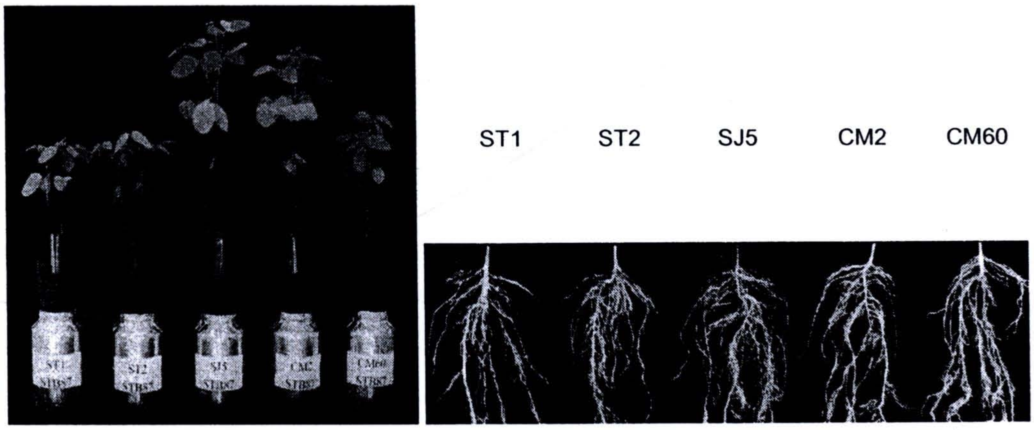
Figure 4.2 Bromthymol blue reactions of some representative of fast- (STB 97) and slow-growing isolates which secreted alkaline product(s) throughout the 10-day incubation period (STB8) and secreted alkali product(s) after 5-day incubation and acidic product(s) was secreted after 10-day incubation (STB 30).

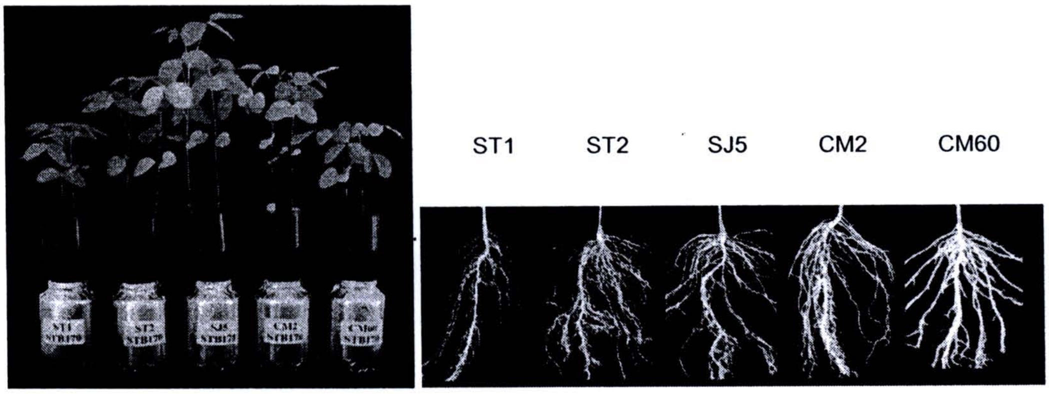
4.4 Authentication test of bacterial isolates from root nodules of soybeans

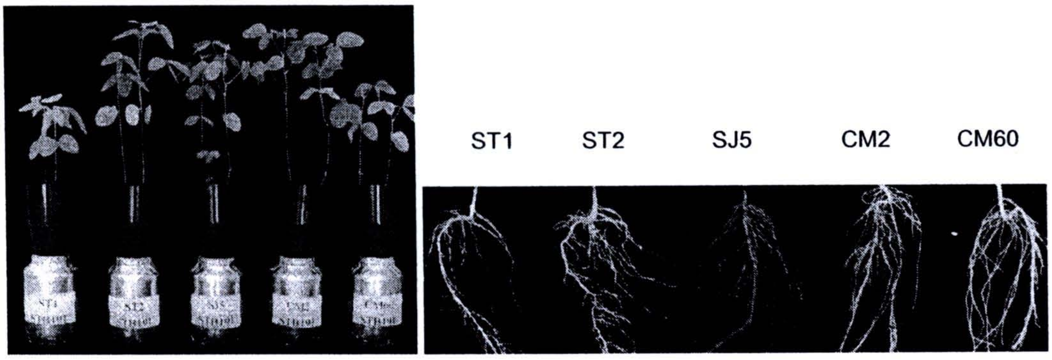
Figures 4.3 and 4.4 showed representative results of authentication test of fast-growing (STB 23, STB 83, STB 87, STB 170, STB 191, STB 192, STB240) and slow-growing (STB 1, STB 5 STB 8, STB 272, STB310) bacterial isolates from root nodules of 5 soybean cultivars grown in soils from 16 subdistricts of Phitsanulok province.

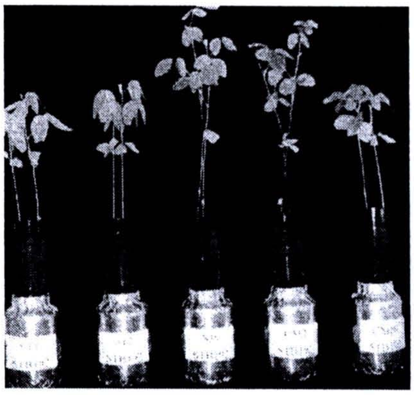


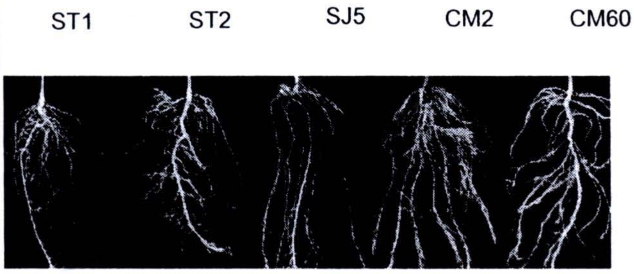

ST1
ST2
SJ5
CM2
CM60

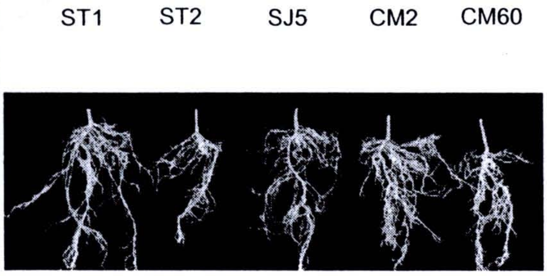

ST1
ST2
SJ5
CM2
CM60


ST1
ST2
SJ5
CM2
CM60

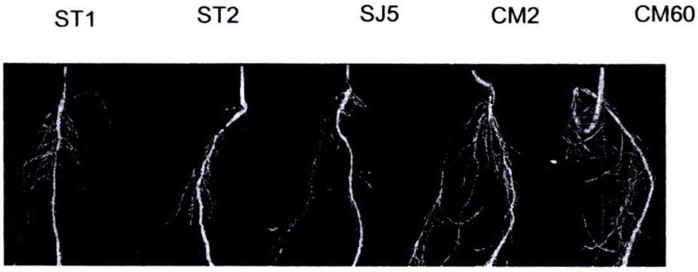

ST1
ST2
SJ5
CM2
CM60

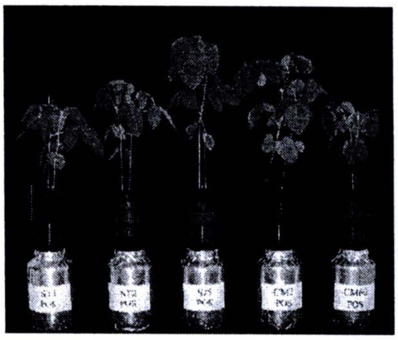






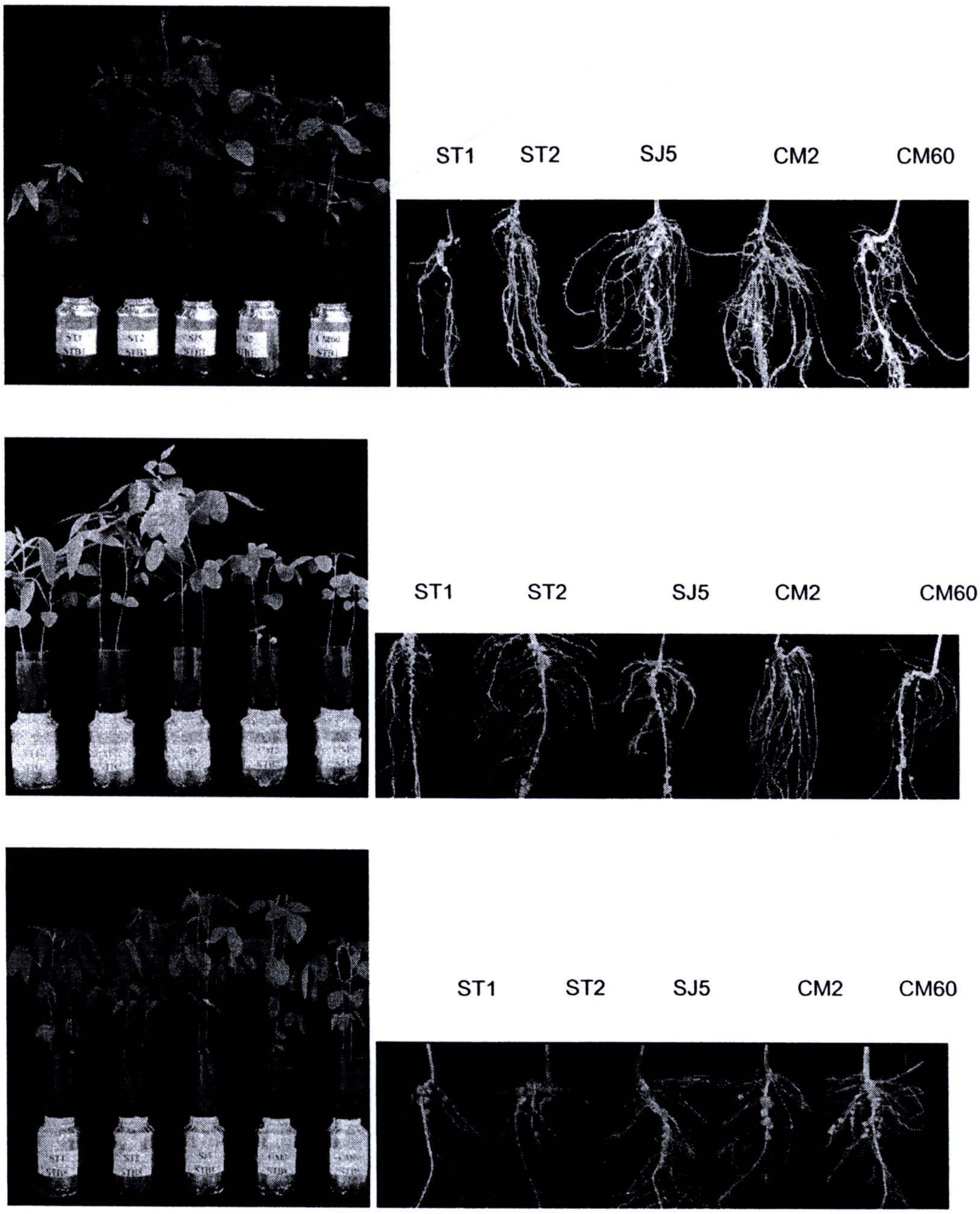
Negative Control:

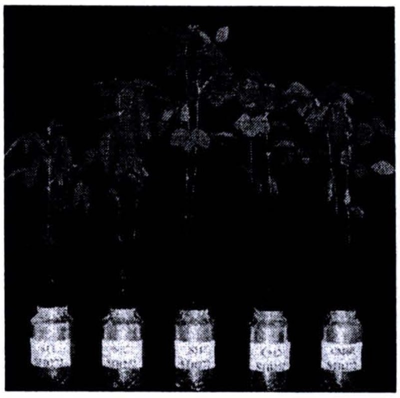


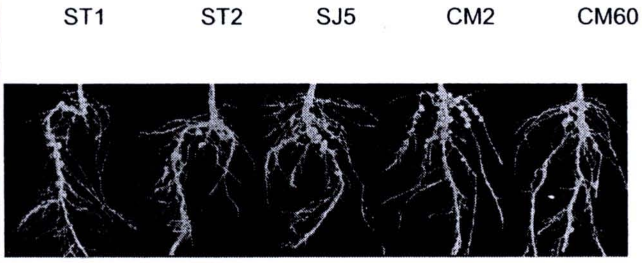


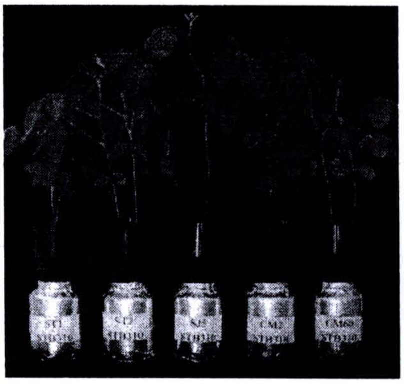
Positive Control:

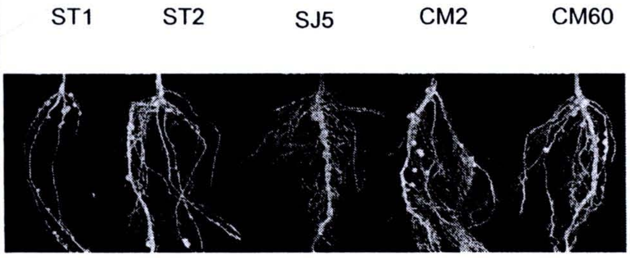
Figures 4.3 Representative authentication test results of fast-growing bacterial isolates (STB 23, STB 83, STB 87, STB 170, STB 191, STB 192, and STB240) grown in Leonard jars with two germinating seeds per jar. Five ml of each bacterial suspension in yeast extract mannnitol broth were added to each germinating seed of soybean cultivars ST1, ST2, SJ5, CM2, and CM60 in Leonard jars which were placed in a 28^{0}C- 32^{0}C temperature-controlled greenhouse for 28 days before the observation of roots. Negative and positive control soybean plants were found to contain no root nodules.











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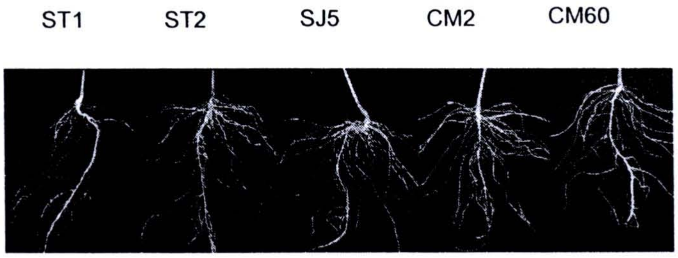
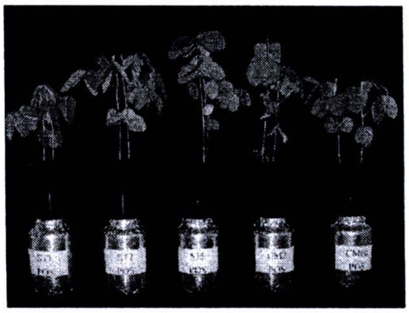




Positive Control:

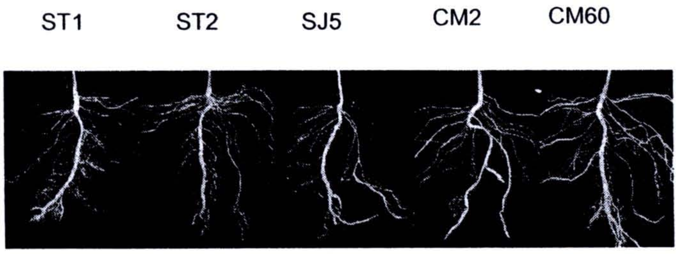


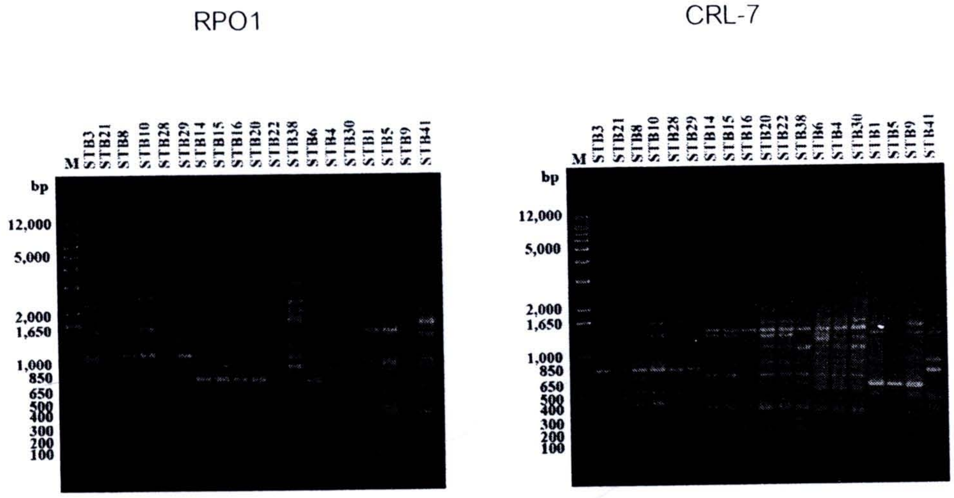
Figure 4.4 Representative authentication test results of slow-growing bacterial isolates (STB1, STB5, STB 8, STB 272, and STB 310) grown in Leonard jars with two germinating seeds per jar. Five ml of each bacterial suspension in yeast extract mannnitol broth were added to each germinating seed of soybean cultivars ST1, ST2, SJ5, CM2, and CM60 in Leonard jars which were placed in a 28^0C- 32^0C temperature-controlled greenhouse for 28 days before the observation of root nodules. Negative and positive control soybean plants were found to contain no root nodules.

Figures 4.3 and 4.4 showed the representative fast-growing bacterial isolates did not cause root nodule formation while the representative slow-growing isolates formed root nodules in all the 5 soybean cultivars used in the experiments. The rationale behind the use of 5 soybean cultivars in the authentication test, especially in the authentication test of fast-growing isolates, was because fast-growing soybean rhizobia had been shown to be relatively more soybean host-specific than slow-growing soybean rhizobia. For example, in 1998 Pueppke et al. reported that fast-growing soybean rhizobium, *Sinorhizobium fredii* strain USDA257 could nodulate soybean *Glycine max* cv. Peking but not cv. McCall while *Sinorhizobium fredii* strain USDA191 could nodulate both soybean cultivars. The researchers employed reverse-phase HPLC and electrospray mass spectrophotometry to determine types and quantities of flavonoids secreted by roots of germinating seeds of each soybean cultivar. The results indicated root exudates of both soybean cultivars contained the same flavonoids genistein, daidzein, and coumestrol with soybean cultivar Peking secreted relatively high concentration of daidzein (average 1371 pmol. seedling^{-1}). The authors suggested that different environments regarding different flavonoid levels in the rhizosphere of soybean cultivars McCall and Peking, and possibly the differential ability to uptake the flavonoid signal molecules could explain different host-specificity in soybean and *S. fredii* system. Therefore, in the authentication experiments, 5 soybean cultivars which had been recommended to soybean growers by the Department of Agricultural Extension, Ministry of Agriculture and Cooperatives, were used to take into account possible specific host requirements of fast-growing soybean rhizobia.

Authentication test results indicated the 138 fast-growing root nodule bacteria did not cause nodule formation in roots of the 5 soybean cultivars. They were found not to be fast-growing soybean rhizobia. On the other hand, all the 202 slow-growing isolates were found to cause nodule formation on roots of all the 5 soybean cultivars grown in Leonard jars in a 28^0 C - 32^0 C temperature-controlled greenhouse. The 202 bacterial root nodule isolates were authenticated to be slow-growing soybean rhizobia.

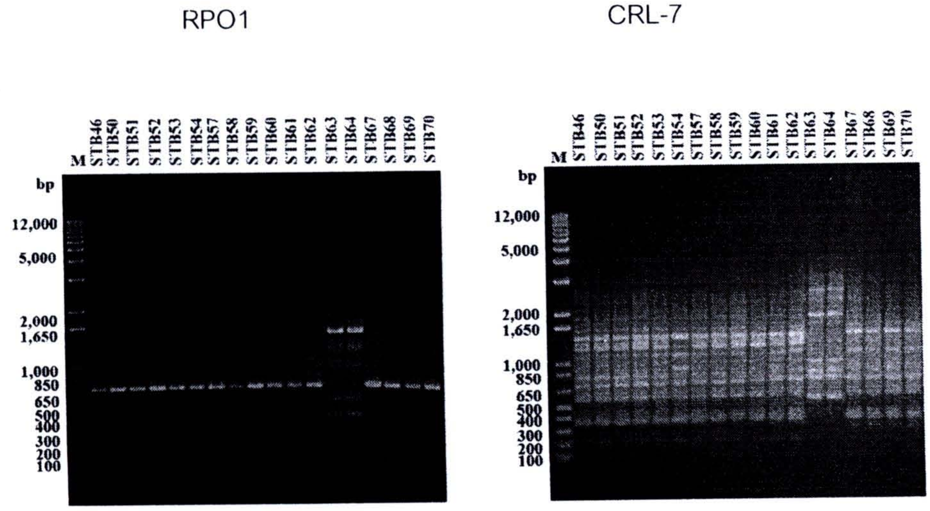
4.5 PCR-DNA fingerprints of 202 slow-growing soybean rhizobia

Figures 4.5-4.16 showed PCR-DNA fingerprints of 202 slow-growing soybean rhizobium isolates obtained from 16 subdistricts in Phitsanulok province. The results showed some isolates had identical fingerprints when either RPO1 or CRL-7 was used as the primer in obtaining the fingerprints. Isolates with identical DNA fingerprints were regarded as the same strains using the lowest code number of the isolates as each strain's code number. For example, isolates STB4 and STB30 had identical DNA fingerprints when either RPO1 or CRL-7 was used as the primer. Therefore the two isolates were regarded as the same strain STB4. Table 4.4 showed isolates with identical DNA fingerprints were grouped into the same strains with a total of 43 strains. Figure 4.17 showed 33 strains with different fingerprints from each other and from other isolates. Therefore the 33 strains constitute distinctive strains. All together, PCR-DNA fingerprints revealed a total of 121 slow-growing soybean rhizobium strains were obtained in this research.


RPO1

CRL-7

Figure 4.5 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Ban Pa and Hua Ro subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB1 = STB5 = STB9, STB3 = STB21, STB4 = STB30, STB8 = STB10 = STB28 = STB29, STB20 = STB22, STB14 = STB15.


RPO1

CRL-7

Figure 4.6 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Wat Phrik and Wang Ithok subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB46 = STB50 = STB51 = STB52 = STB53 = STB57 = STB58 = STB59 = STB60 = STB61 = STB62, STB63 = STB64, STB67 = STB68 = STB69 = STB70.

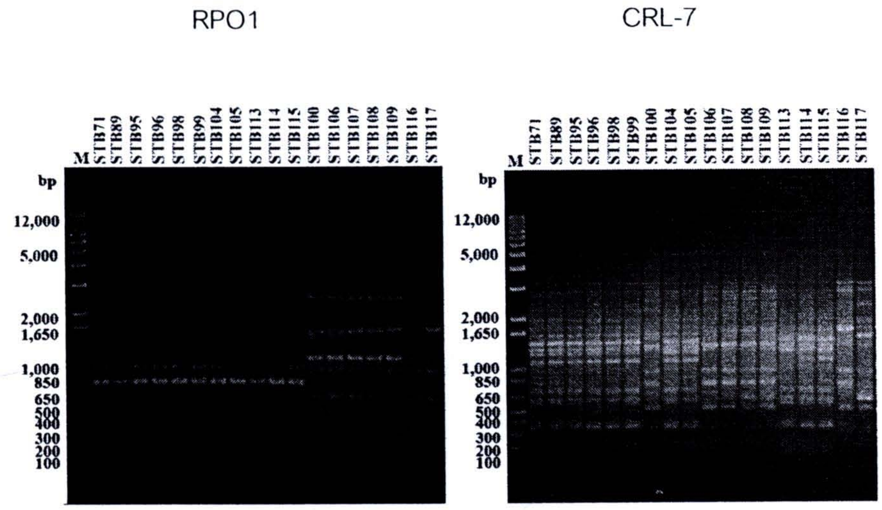


Figure 4.7 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Wang Ithok, Mathong and Sri Phirom subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB71= STB95 = STB96 = STB98 = STB99 = STB104 = STB105 = STB115, STB100 = STB106 = STB107 = STB108 = STB109.

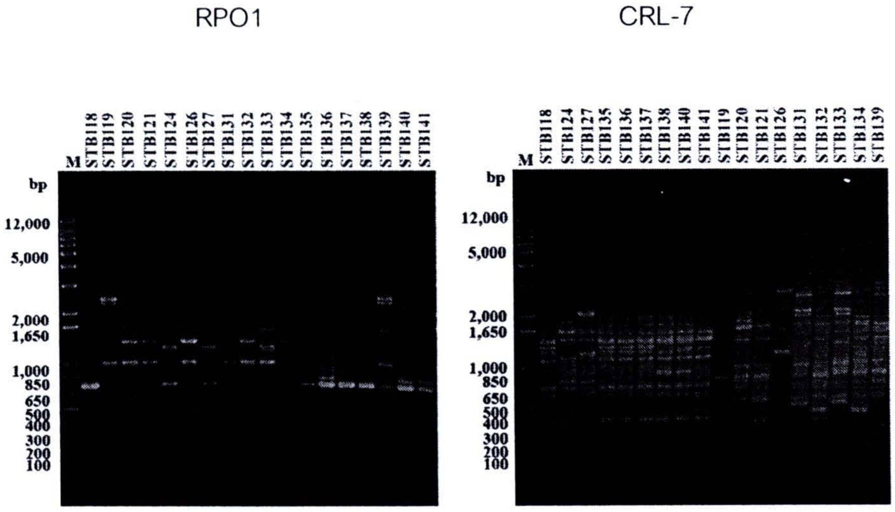


Figure 4.8 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Sri Phirom and Tha Chang subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB118 = STB135 = STB136 = STB137, STB131 = STB133, STB132 = STB134, STB138 = STB141.

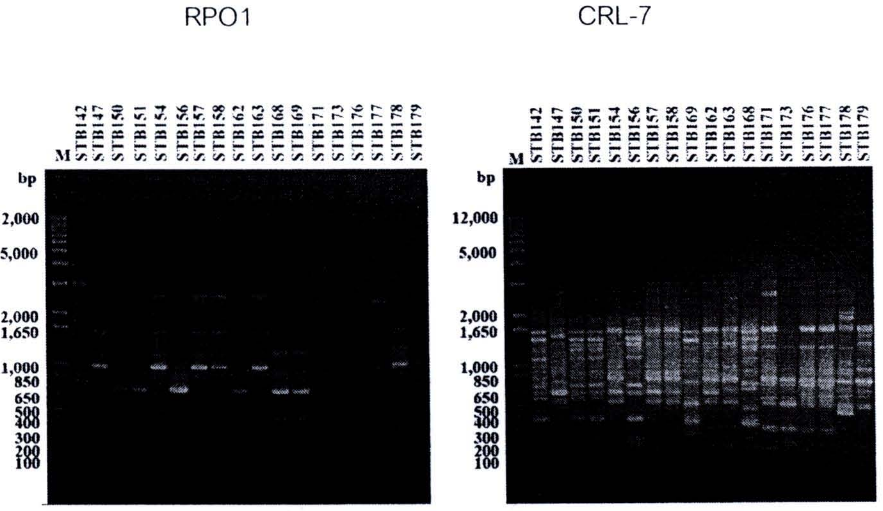


Figure 4.9 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Tha Chang and Ban Dong subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB150 = STB151 = STB156, STB154 = STB157 = STB158, STB171 = STB176 = STB177.

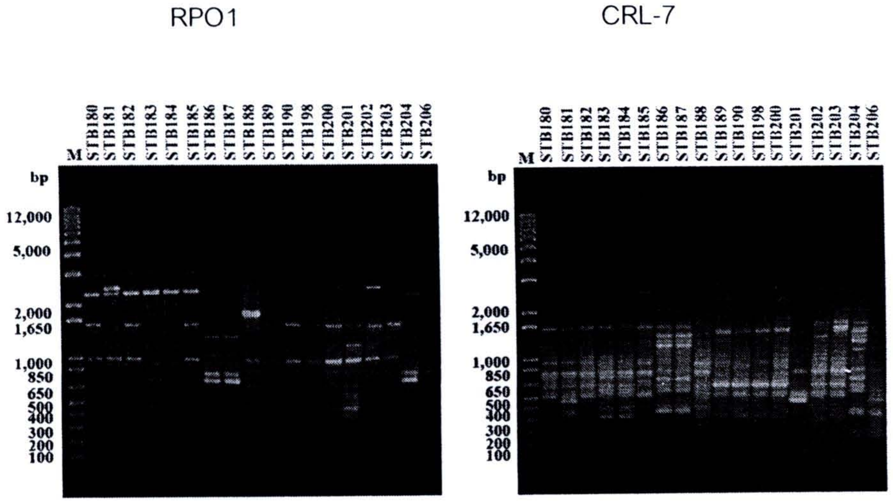


Figure 4.10 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Ban Dong, Ban Phrao and Nakhon Chum subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB180 = STB182, STB189 = STB190 = STB198 = STB200, STB183 = STB184, STB186 = STB187 = STB204.

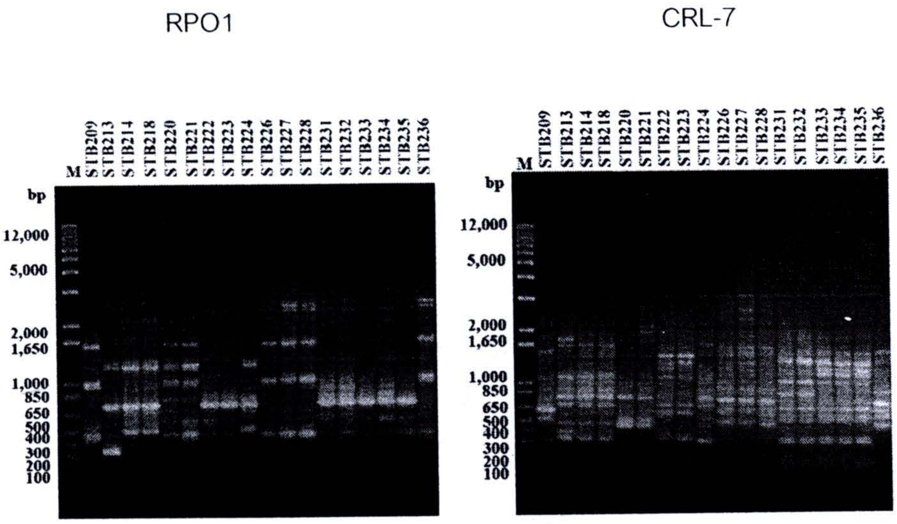


Figure 4.11 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Nakhon Chum and Nong Kathao subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB214 = STB218 , STB220 = STB221, STB222 = STB223, STB226 = STB227 = STB228, STB231 = STB232, STB233 = STB235.

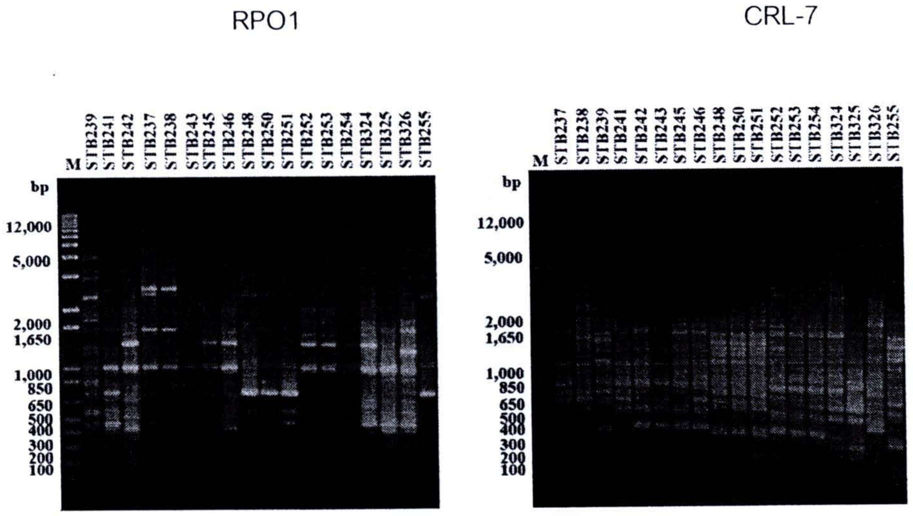


Figure 4.12 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Nong Kathao and Chaiyanam subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB248 = STB251, STB252 = STB253, STB242 = STB245 = STB246, STB252 = STB253 = STB 324.

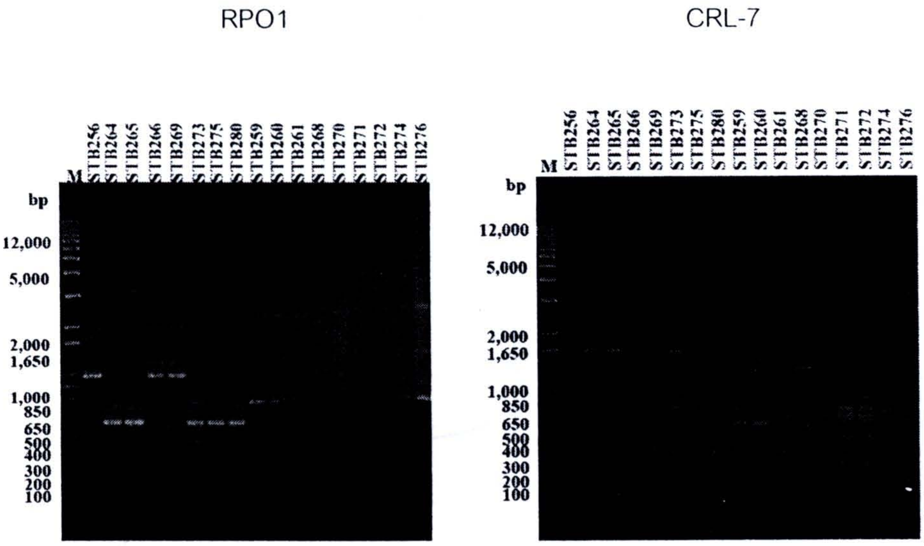


Figure 4.13 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Chaiyanam and Kang Sopha subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB259 = STB260, STB264 = STB265 = STB280, STB266 = STB269, STB271 = STB272.

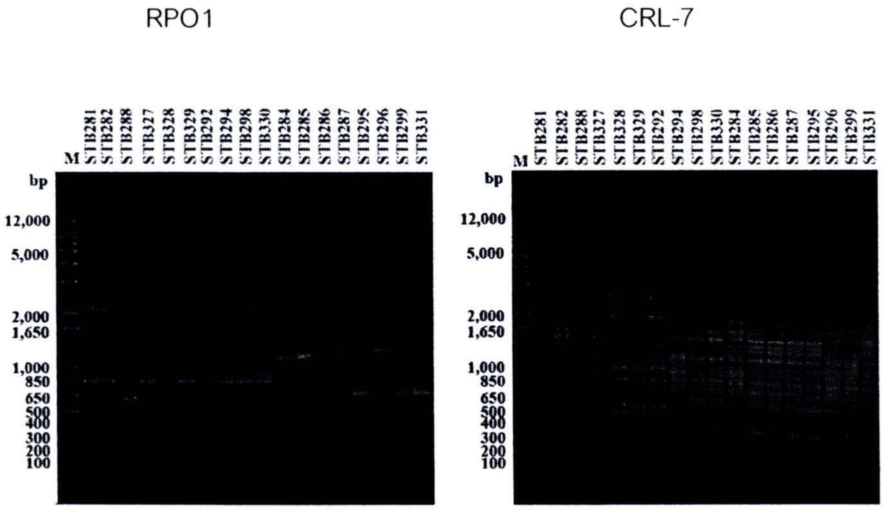
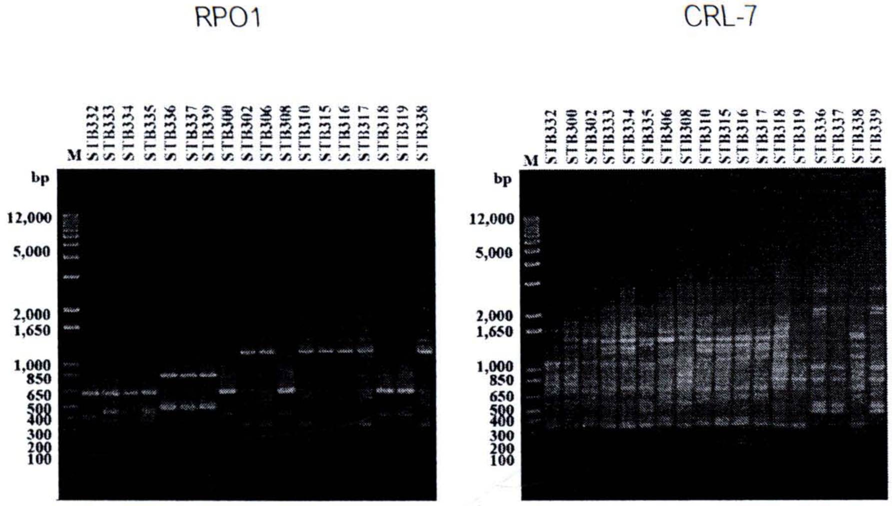


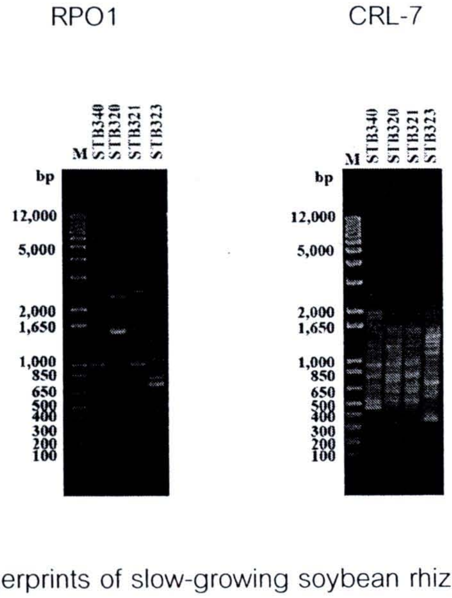
Figure 4.14 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Kang Sopha and Nong Phra subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB299 = STB331, STB286 = STB287.



RPO1

CRL-7

Figure 4.15 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Nong Phra and Tha Muen Ram subdistricts, Phitsanulok province. The results showed the following isolates were the same strains: STB300 = STB308, STB302 = STB306, STB310 = STB315 = STB316 = STB317 = STB338, STB333 = STB334, STB336 = STB337 = STB339.

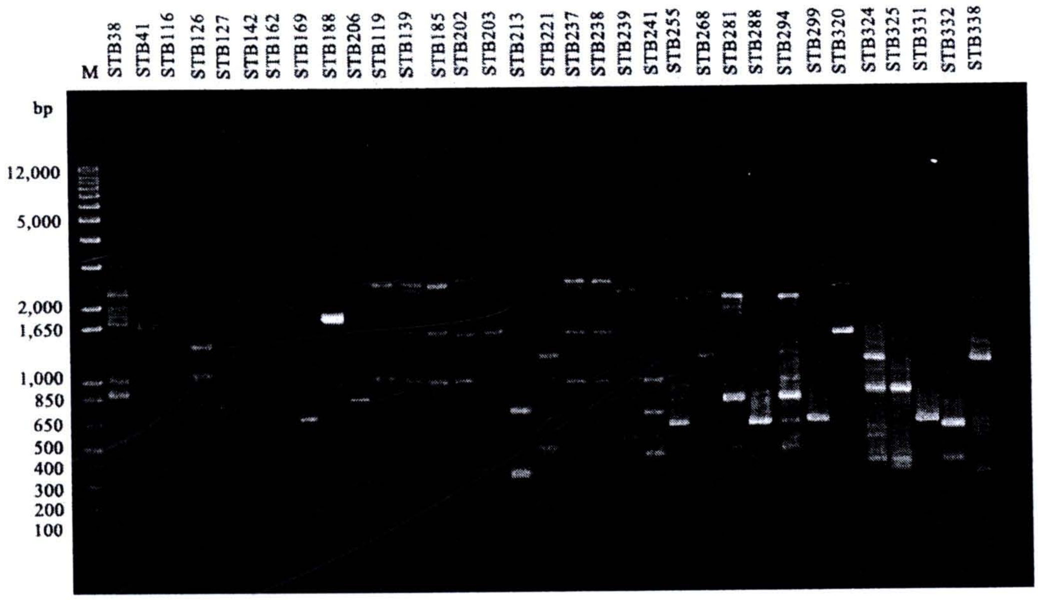


RPO1

CRL-7

Figure 4.16 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from Tha Muen Ram subdistrict, Phitsanulok province.

RPO1



CRL-7

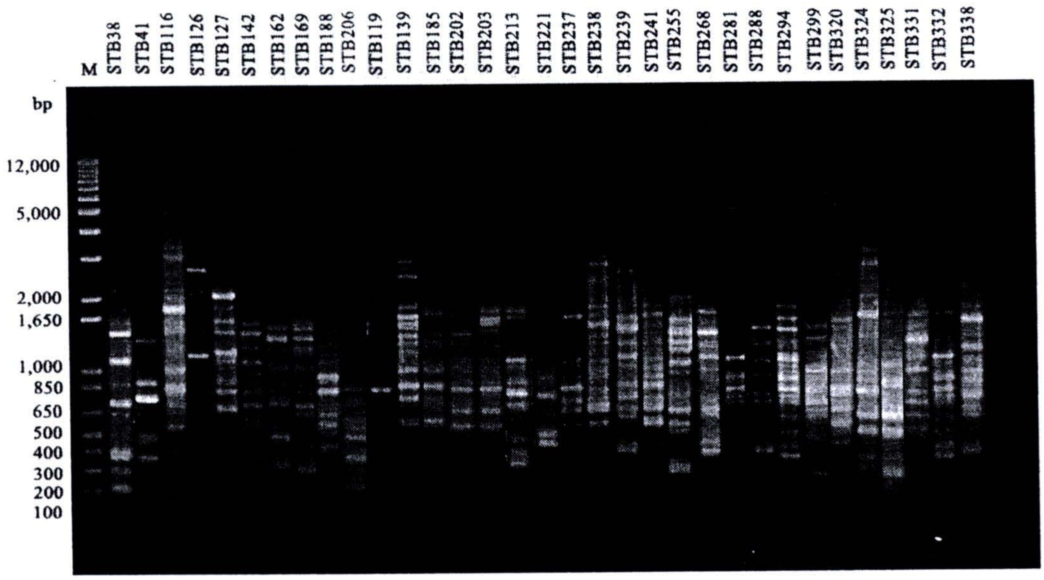


Figure 4.17 PCR-DNA fingerprints of slow-growing soybean rhizobium isolates obtained from several subdistricts of Phitsanulok province which have distinct individual fingerprints.

Table 4.4 Slow-growing soybean rhizobium isolates with identical RAPD-PCR fingerprints were grouped into the same strains.

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB1	STB1	Ban Pa	ST 1
	STB5	Ban Pa	ST 2
	STB9	Ban Pa	SJ 5
STB3	STB3	Ban Pa	ST1
	STB21	Hua Ro	ST 1
STB4	STB4	Ban Pa	ST1
	STB30	Hua Ro	SJ 5
STB6	STB6	Ban Pa	ST2
STB8	STB8	Ban Pa	SJ 5
	STB10	Ban Pa	SJ 5
STB14	STB14	Ban Pa	CM60
	STB15	Ban Pa	CM60
STB16	STB16	Ban Pa	CM60
STB20	STB20	Ban Pa	CM60
	STB22	Hua Ro	ST1
STB28	STB28	Hua Ro	ST2
	STB29	Hua Ro	ST 2
STB38	STB38	Hua Ro	CM2
STB41	STB41	Hua Ro	CM2
STB46	STB46	Wat Phrik	ST1
	STB50	Wat Phrik	ST2
	STB51	Wat Phrik	ST2
	STB52	Wat Phrik	ST2
	STB53	Wat Phrik	ST2
	STB57	Wat Phrik	SJ5

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
	STB58	Wat Phrik	SJ5
	STB59	Wat Phrik	SJ5
	STB60	Wat Phrik	CM2
	STB61	Wat Phrik	CM2
	STB62	Wat Phrik	CM2
STB54	STB54	Wat Phrik	ST2
STB63	STB63	Wat Phrik	CM2
	STB64	Wat Phrik	CM2
STB67	STB67	Wang Ithok	ST1
	STB68	Wang Ithok	ST1
	STB69	Wang Ithok	ST1
	STB70	Wang Ithok	ST1
STB71	STB71	Wang Ithok	ST2
	STB95	Mathong	SJ5
	STB96	Mathong	CM2
	STB98	Mathong	SJ 5
	STB99	Mathong	ST2
	STB104	Mathong	CM2
	STB105	Mathong	CM2
	STB115	Sri Phirom	ST1
STB89	STB89	Wang Ithok	ST2
STB100	STB100	Mathong	SJ 5
	STB106	Mathong	CM2
	STB107	Mathong	CM2
	STB108	Mathong	CM60
	STB109	Mathong	SJ5
STB113	STB113	Sri Phirom	CM60

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB114	STB114	Sri Phirom	CM2
STB116	STB116	Mathong	ST2
STB117	STB117	Sri Phirom	ST2
STB118	STB118	Sri Phirom	SJ5
	STB135	Tha Chang	SJ5
	STB136	Tha Chang	CM60
	STB137	Tha Chang	CM2
STB119	STB119	Mathong	ST2
STB120	STB120	Sri Phirom	SJ5
STB121	STB121	Sri Phirom	SJ5
STB124	STB124	Sri Phirom	CM2
STB126	STB126	Sri Phirom	CM2
STB127	STB127	Sri Phirom	CM2
STB131	STB131	Sri Phirom	ST2
	STB133	Sri Phirom	ST2
STB132	STB132	Sri Phirom	CM60
	STB134	Sri Phirom	CM60
STB138	STB138	Tha Chang	SJ5
	STB141	Tha Chang	CM2
STB139	STB139	Tha Chang	ST2
STB140	STB140	Tha Chang	CM2
STB142	STB142	Tha Chang	ST2
STB147	STB147	Tha Chang	CM2
STB150	STB150	Tha Chang	CM60
	STB151	Tha Chang	CM60
	STB156	Ban Dong	ST1
STB154	STB154	Ban Dong	CM2

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
	STB157	Ban Dong	CM2
	STB158	Ban Dong	ST2
STB162	STB162	Ban Dong	ST2
STB163	STB163	Ban Dong	ST1
STB168	STB168	Ban Dong	CM2
STB169	STB169	Ban Dong	CM2
STB171	STB171	Ban Dong	CM2
	STB176	Ban Dong	CM2
	STB177	Ban Dong	CM2
STB173	STB173	Ban Dong	CM2
STB178	STB178	Ban Dong	CM60
STB179	STB179	Ban Dong	CM60
STB180	STB180	Ban Dong	CM60
	STB182	Ban Dong	CM60
STB181	STB181	Ban Dong	CM60
STB183	STB183	Ban Dong	CM60
	STB184	Ban Dong	CM60
STB185	STB185	Ban Dong	CM60
STB186	STB186	Ban Phrao	ST1
	STB187	Ban Phrao	ST1
	STB204	Nakhon Chum	ST1
STB188	STB188	Ban Dong	ST2
STB189	STB189	Ban Phrao	ST 2
	STB190	Ban Phrao	ST 2
	STB198	Ban Phrao	CM2
	STB200	Ban Phrao	CM2
STB201	STB201	Ban Phrao	CM60

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB202	STB202	Ban Dong	CM60
STB203	STB203	Ban Dong	CM60
STB206	STB206	Nakhon Chum	ST2
STB209	STB209	Nakhon Chum	ST 2
STB213	STB213	Nakhon Chum	ST2
STB214	STB214	Nakhon Chum	ST2
	STB218	Nakhon Chum	SJ5
STB220	STB220	Nakhon Chum	CM2
	STB221	Na Khon Chum	CM2
STB222	STB222	Na Khon Chum	ST1
	STB223	Na Korn Chum	ST2
STB224	STB224	Nakhon Chum	CM60
STB226	STB226	Nong Kathao	ST1
	STB227	Nong Kathao	ST1
	STB228	Nong Kathao	ST1
STB231	STB231	Nong Kathao	ST2
	STB232	Nong Kathao	ST2
STB233	STB233	Nong Kathao	SJ5
	STB235	Nong Kathao	ST2
STB234	STB234	Nong Kathao	SJ5
STB236	STB236	Nong Kathao	SJ5
STB237	STB237	Nong Kathao	SJ5
STB238	STB238	Nong Kathao	SJ5
STB241	STB241	Nong Kathao	CM2
STB242	STB242	Nong Kathao	CM2
	STB245	Nong Kathao	ST1
	STB246	Nong Kathao	ST1

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB243	STB243	Nong Kathao	CM2
STB248	STB248	Nong Kathao	CM2
	STB251	Chaiyanam	CM2
STB250	STB250	Ban Dong	CM2
STB252	STB252	Chaiyanam	CM60
	STB253	Chaiyanam	ST2
	STB324	Chaiyanam	SJ5
STB254	STB254	Chaiyanam	ST2
STB255	STB255	Chaiyanam	CM2
STB256	STB256	Chaiyanam	CM2
STB259	STB259	Chaiyanam	CM60
	STB260	Chaiyanam	CM60
STB264	STB264	Kang Sopha	ST1
	STB265	Kang Sopha	ST1
	STB280	Kang Sopha	CM60
STB261	STB261	Chaiyanam	CM60
STB266	STB266	Kang Sopha	ST2
	STB269	Kang Sopha	ST2
STB268	STB268	Kang Sopha	ST2
STB270	STB270	Kang Sopha	SJ5
STB271	STB271	Kang Sopha	SJ5
	STB272	Kang Sopha	SJ5
STB273	STB273	Kang Sopha	CM2
STB274	STB274	Kang Sopha	CM2
STB275	STB275	Kang Sopha	CM2
STB276	STB276	Kang Sopha	CM60
STB281	STB281	Mae Raka	ST1

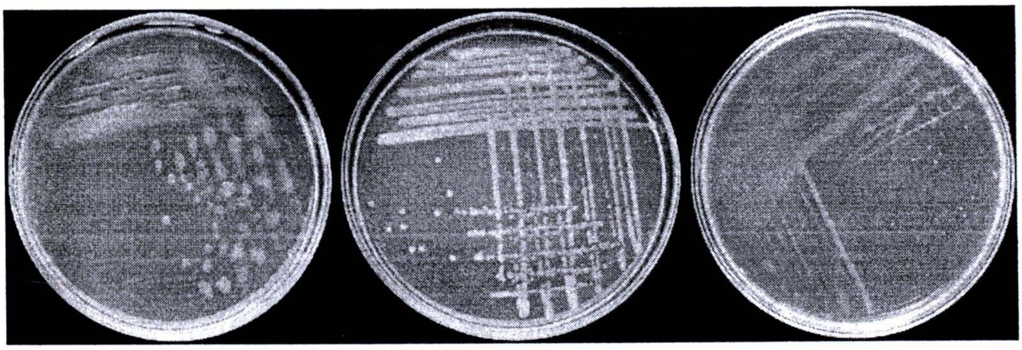
Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB282	STB282	Mae Raka	ST2
STB284	STB284	Mae Raka	CM2
STB285	STB285	Mae Raka	ST2
STB286	STB286	Mae Raka	ST2
	STB287	Mae Raka	ST2
STB288	STB288	Mae Raka	ST2
STB292	STB292	Mae Raka	CM2
STB294	STB294	Mae Raka	CM60
STB295	STB295	Nong Phra	SJ5
STB296	STB296	Nong Phra	CM2
STB298	STB298	Nong Phra	ST1
STB299	STB299	Nong Phra	ST1
	STB331	Nong Phra	ST2
STB300	STB300	Nong Phra	SJ5
	STB308	Nong Phra	CM60
STB302	STB302	Nong Phra	SJ5
	STB306	Nong Phra	CM60
STB310	STB310	Ta Muen Ram	ST1
	STB315	Ta Muen Ram	ST2
	STB316	Ta Muen Ram	ST2
	STB317	Ta Muen Ram	ST2
	STB338	Ta Muen Ram	SJ5
STB318	STB318	Ta Muen Ram	ST2
STB319	STB319	Ta Muen Ram	ST2
STB320	STB320	Tha Muen Ram	CM60
STB321	STB321	Ban Dong	ST1
STB323	STB323	Ta Muen Ram	CM60

Strains	Isolates	Sources	
		Soil sample subdistricts	Root nodules of soybean cultivars
STB325	STB325	Chaiyanam	SJ5
STB326	STB326	Chaiyanam	SJ5
STB327	STB327	Mae Raka	CM2
STB328	STB328	Mae Raka	ST1
STB329	STB329	Mae Raka	ST1
STB330	STB330	Nong Phra	ST2
STB332	STB332	Nong Phra	ST2
STB333	STB333	Nong Phra	CM2
	STB334	Nong Phra	CM2
STB336	STB336	Kang Sopha	CM60
	STB337	Ta Muen Ram	SJ5
	STB339	Ta Muen Ram	SJ5
STB335	STB335	Nong Phra	CM2
STB340	STB340	Tha Muen Ram	CM2

4.6 Polyphasic characterization of slow-growing soybean rhizobia

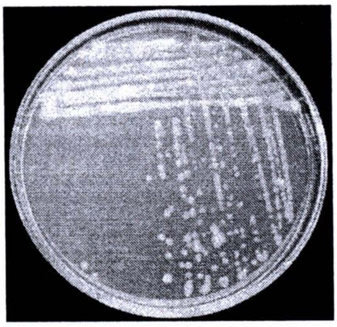
4.6.1 Colony morphology

Figure 4.18 showed representative colony morphology of 4 slow-growing soybean rhizobium strains. The results indicated Type I colonies were irregular and slimy after 10- day incubation on YMA with congo red medium. Type II colonies were round and pearly. Type III colonies were round and shiny with very small colonies (diameter less than 0.01 mm) while Type IV colonies were round and slimy. All the slow-growing soybean rhizobia were found not to absorb congo red.



STB 8 STB96 STB336

Type I colony Type II colony Type III colony



STB264

Type IV colony

Figure 4.18 Colony morphology of representative slow-growing soybean rhizobia isolated from soils from 16 subdistricts in Phitsanulok province. Type I colonies appeared irregular and slimy. Type II colonies appeared round and pearly. Type III colonies were round and shiny with very small colonies (diameter less than 0.01 mm). Type IV colonies were round and slimy.

4.6.2 Bromthymol blue reactions

Table 4.5 showed colony morphology and Bromthymol blue (BTB) reactions of the 121 slow-growing soybean rhizobium strains. The results of BTB reactions showed most of the soybean rhizobia with Type I colonies secreted alkali product(s) throughout the 10-day incubation period except STB1, STB117, STB209, STB274, STB319, STB328, STB330, and STB335. Soybean rhizobia with Type II colonies secreted alkali product(s) after 5-day incubation and secreted acidic product(s) after 10-day incubation except STB285, STB286, and STB295. All soybean rhizobia of Type III colony with the smallest colonies which were less than 0.01 mm in diameter (STB28, STB41, STB116, STB139, STB162, STB163, STB188, STB321, STB326, STB336, and STB340) except STB168 were found to secrete alkali product(s) throughout the 10-day incubation period. Type IV colonies (STB264 and STB302) were found to secrete alkali product(s) after 5-day incubation and secrete acidic product(s) after 10-day incubation.

Table 4.5 Colony morphology and BTB reactions of 121 strains of slow-growing soybean rhizobia isolated from soils from 16 subdistricts of Phitsanulok province. (+ = H^+ secretion; - = OH^- secretion).

Strain no.	Code	Subdistrict	Type of colony	Bromthymol blue reactions after incubation for	
				5 days	10 days
1	STB1	Ban Pa	I	-	+
2	STB3	Ban Pa	I	-	-
3	STB4	Ban Pa	II	-	+
4	STB6	Ban Pa	II	-	+
5	STB8	Ban Pa	I	-	-
6	STB14	Ban Pa	II	-	+
7	STB16	Ban Pa	II	-	+
8	STB20	Ban Pa	II	-	+
9	STB28	Hua Ro	III	-	-

10	STB38	Hua Ro	II	-	+
11	STB41	Hua Ro	III	-	-
12	STB46	Wat Phrik	II	-	+
13	STB54	Wat Phrik	II	-	+
14	STB63	Wat Phrik	I	-	-
15	STB67	Wang Ithok	II	-	+
16	STB71	Wang Ithok	II	-	+
17	STB89	Wang Ithok	II	-	+
18	STB100	Mathong	I	-	-
19	STB113	Sri Phirom	II	-	+
20	STB114	Sri Phirom	II	-	+
21	STB116	Sri Phirom	III	-	-
22	STB117	Sri Phirom	I	-	+
23	STB118	Sri Phirom	II	-	+
24	STB119	Sri Phirom	I	-	-
25	STB120	Sri Phirom	I	-	-
26	STB121	Sri Phirom	I	-	-
27	STB124	Sri Phirom	II	-	+
28	STB126	Sri Phirom	I	-	-
29	STB127	Sri Phirom	II	-	+
30	STB131	Sri Phirom	I	-	-
31	STB132	Sri Phirom	I	-	-
32	STB138	Tha Chang	II	-	+
33	STB139	Tha Chang	III	-	-
34	STB140	Tha Chang	II	-	+
35	STB142	Tha Chang	II	-	+
36	STB147	Tha Chang	I	-	-
37	STB150	Tha Chang	II	-	+
38	STB154	Ban Dong	I	-	-
39	STB162	Ban Dong	III	-	-

40	STB163	Ban Dong	III	-	-
41	STB168	Ban Dong	III	-	+
42	STB169	Ban Dong	I	-	-
43	STB171	Ban Dong	I	-	-
44	STB173	Ban Dong	I	-	-
45	STB178	Ban Dong	I	-	-
46	STB179	Ban Dong	I	-	-
47	STB180	Ban Dong	I	-	-
48	STB181	Ban Dong	I	-	-
49	STB183	Ban Dong	I	-	-
50	STB185	Ban Dong	I	-	-
51	STB186	Ban Phrao	I	-	-
52	STB188	Ban Phrao	III	-	-
53	STB189	Ban Phrao	I	-	-
54	STB201	Ban Phrao	I	-	-
55	STB202	Ban Phrao	I	-	-
56	STB203	Ban Phrao	I	-	-
57	STB206	Nakhon Chum	II	-	+
58	STB209	Nakhon Chum	I	-	+
59	STB213	Nakhon Chum	II	-	+
60	STB214	Nakhon Chum	II	-	+
61	STB220	Nakhon Chum	I	-	-
62	STB222	Nakhon Chum	II	-	+
63	STB224	Nakhon Chum	II	-	+
64	STB226	Nong Kathao	I	-	-
65	STB231	Nong Kathao	II	-	+
66	STB233	Nong Kathao	II	-	+
67	STB234	Nong Kathao	II	-	+
68	STB236	Nong Kathao	I	-	-
69	STB237	Nong Kathao	I	-	-

70	STB238	Nong Kathao	I	-	-
71	STB241	Nong Kathao	I	-	-
72	STB242	Nong Kathao	I	-	-
73	STB243	Nong Kathao	I	-	-
74	STB248	Nong Kathao	II	-	+
75	STB250	Nong Kathao	II	-	+
76	STB252	Chaiyanam	I	-	-
77	STB254	Chaiyanam	I	-	-
78	STB255	Chaiyanam	II	-	+
79	STB256	Chaiyanam	II	-	+
80	STB259	Chaiyanam	I	-	-
81	STB259	Chaiyanam	I	-	-
82	STB264	Kaeng Sopha	IV	-	+
83	STB266	Kaeng Sopha	II	-	+
84	STB268	Kaeng Sopha	I	-	-
85	STB270	Kaeng Sopha	I	-	-
86	STB271	Kang Sopha	I	-	-
87	STB273	Kang Sopha	II	-	+
88	STB274	Kang Sopha	I	-	+
89	STB275	Kang Sopha	II	-	+
90	STB276	Kang Sopha	I	-	-
91	STB281	Mae Raka	I	-	-
92	STB282	Mae Raka	I	-	-
93	STB284	Mae Raka	II	-	+
94	STB285	Mae Raka	II	-	-
95	STB286	Mae Raka	II	-	-
96	STB288	Mae Raka	II	-	+
97	STB292	Mae Raka	I	-	-
98	STB294	Mae Raka	I	-	-
99	STB295	Nong Phra	II	-	-

100	STB296	Nong Phra	II	-	+
101	STB298	Nong Phra	I	-	-
102	STB299	Nong Phra	II	-	+
103	STB300	Nong Phra	II	-	+
104	STB302	Nong Phra	IV	-	+
105	STB310	Tha Muen Ram	II	-	+
106	STB318	Tha Muen Ram	II	-	+
107	STB319	Ta Muen Ram	I	-	+
108	STB320	Tha Muen Ram	I	-	-
109	STB321	Tha Muen Ram	III	-	-
110	STB323	Ta Muen Ram	II	-	+
111	STB325	Chaiyanam	I	-	-
112	STB326	Chaiyanam	III	-	-
113	STB327	Mae Raka	I	-	-
114	STB328	Mae Raka	I	-	+
115	STB329	Mae Raka	I	-	-
116	STB330	Nong Phra	I	-	+
117	STB332	Nong Phra	I	-	-
118	STB333	Nong Phra	I	-	-
119	STB335	Nong Phra	I	-	+
120	STB336	Tha Muen Ram	III	-	-
121	STB340	Tha Muen Ram	III	-	-

The observed ability of *B. elkanii* strain NA7, *B. japonicum* strain S76, *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 to secrete either acidic or alkali product(s) in response to surrounding pHs were shown in Tables 4.6- 4.13.

Table 4.6 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM NEDA buffer at the initial pH of 4.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM NEDA			pH in YMB without 30mM NEDA		
		1	2	average	1	2	average
B.elkanii NA7	1	3.94	3.94	3.94	5.57	5.52	5.55
	2	3.98	3.98	3.98	5.74	5.72	5.73
	3	3.98	3.98	3.98	5.32	5.34	5.33
	4	3.98	3.98	3.98	4.50	4.63	4.57
	5	3.99	4.00	4.00	4.31	4.27	4.29
B. japonicum	1	3.94	3.94	3.94	5.55	5.61	5.58
S76	2	3.99	3.98	3.99	5.91	5.90	5.91
	3	3.98	3.98	3.98	5.82	5.83	5.83
	4	3.98	3.98	3.98	5.60	5.95	5.78
	5	3.99	3.99	3.99	5.52	4.70	5.11

The results as shown in Table 4.6 indicated that in the presence of 30 mM NEDA buffer, pH of YMB medium was maintained close to the initial pH of 4.0. However, when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.7 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM NEDA buffer at the initial pH of 4.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM NEDA			pH in YMB without 30mM NEDA		
		1	2	average	1	2	average
B. liaoningense	1	3.98	3.98	3.98	4.80	5.40	5.10
SK3	2	3.98	3.99	3.99	5.39	5.19	5.29
	3	4.00	4.00	4.00	5.43	5.52	5.48
	4	4.00	3.99	4.00	5.67	5.49	5.58
	5	3.99	3.99	3.99	4.62	5.55	5.09
B. yuanmingense	1	3.98	3.98	3.98	4.54	4.61	4.58
STB264	2	3.98	3.98	3.98	4.47	4.60	4.54
	3	3.99	3.99	3.99	5.36	5.06	5.21
	4	3.99	3.99	3.99	5.66	4.61	5.14
	5	3.99	3.99	3.99	5.40	5.55	5.48

The results as shown in Table 4.7 indicated that in the presence of 30 mM NEDA buffer, pH of YMB medium was maintained close to the initial pH of 4.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.8 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM NEDA buffer at the initial pH of 5.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM NEDA			pH in YMB without 30mM NEDA		
		1	2	average	1	2	average
B.elkanii NA7	1	4.96	4.96	4.96	6.29	6.30	6.30
	2	4.99	4.99	4.99	6.32	6.31	6.32
	3	5.00	4.99	5.00	6.11	6.12	6.12
	4	5.01	5.02	5.02	5.82	5.75	5.79
	5	5.02	5.03	5.03	5.52	5.50	5.51
B. japonicum	1	4.97	4.98	4.98	6.25	6.25	6.25
S76	2	5.00	4.99	5.00	6.40	6.38	6.39
	3	5.00	5.00	5.00	6.34	6.35	6.35
	4	5.01	5.02	5.02	6.37	6.28	6.33
	5	5.01	5.02	5.02	6.16	6.20	6.18

The results as shown in Table 4.8 indicated that in the presence of 30 mM NEDA buffer, pH of YMB medium was maintained close to the initial pH of 5.0. However, when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.9 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM NEDA buffer at the initial pH of 5.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM NEDA			pH in YMB without 30mM NEDA		
		1	2	average	1	2	average
B. liaoningense	1	5.03	5.03	5.03	6.37	6.43	6.40
SK3	2	5.04	5.03	5.04	6.33	6.26	6.30
	3	5.03	5.04	5.04	6.21	6.21	6.21
	4	5.03	5.02	5.03	6.27	6.04	6.16
	5	5.02	5.03	5.03	5.98	5.97	5.98
B. yuanmingense	1	5.01	5.02	5.02	6.27	6.25	6.26
STB264	2	5.03	5.02	5.03	6.37	6.35	6.36
	3	5.03	5.03	5.03	6.27	6.21	6.24
	4	5.03	5.03	5.03	6.20	6.12	6.16
	5	5.02	5.02	5.02	6.25	6.13	6.19

The results as shown in Table 4.9 indicated that in the presence of 30 mM NEDA buffer, pH of YMB medium was maintained close to the initial pH of 5.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.10 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM MES buffer at the initial pH of 6.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM MES			pH in YMB without 30mM MES		
		1	2	average	1	2	average
B.elkanii NA7	1	6.03	6.03	6.03	6.51	6.46	6.49
	2	6.07	6.07	6.07	6.62	6.61	6.62
	3	6.06	6.05	6.06	6.49	6.53	6.51
	4	6.05	6.05	6.05	6.29	6.47	6.38
	5	6.05	6.04	6.05	6.26	6.29	6.28
B. japonicum	1	6.04	6.04	6.04	6.47	6.47	6.47
S76	2	6.06	6.06	6.06	6.60	6.55	6.58
	3	6.06	6.06	6.06	6.59	6.57	6.58
	4	6.06	6.06	6.06	6.43	6.56	6.50
	5	6.05	6.06	6.06	6.54	6.52	6.53

The results as shown in Table 4.10 indicated that in the presence of 30 mM MES buffer, pH of YMB medium was maintained close to the initial pH of 6.0. However, when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.11 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM MES buffer at the initial pH of 6.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM MES			pH in YMB without 30mM MES		
		1	2	average	1	2	average
B. liaoningense	1	6.08	6.08	6.08	6.70	6.68	6.69
SK3	2	6.09	6.09	6.09	6.61	6.61	6.61
	3	6.08	6.08	6.08	6.57	6.54	6.56
	4	6.09	6.08	6.09	6.62	6.66	6.64
	5	6.06	6.07	6.07	6.65	6.65	6.65
B. yuanmingense	1	6.08	6.08	6.08	6.69	6.67	6.68
STB264	2	6.09	6.09	6.09	6.64	6.61	6.63
	3	6.08	6.09	6.09	6.62	6.55	6.59
	4	6.07	6.08	6.08	6.52	6.52	6.52
	5	6.05	6.07	6.06	6.60	6.60	6.60

The results as shown in Table 4.11 indicated that in the presence of 30 mM MES buffer, pH of YMB medium was maintained close to the initial pH of 6.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete alkali product(s) to increase pHs of the medium to a less acidic range.

Table 4.12 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM HEPES buffer at the initial pH of 7.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM HEPES			pH in YMB without 30mM HEPES		
		1	2	average	1	2	average
B.elkanii NA7	1	7.08	7.08	7.08^{abcde}	7.12	7.13	7.13^{ab}
	2	7.04	7.05	7.05^{cdefg}	7.14	7.14	7.14^{a}
	3	7.01	7.03	7.02^{efg}	7.11	7.10	7.11^{abc}
	4	7.01	7.01	7.01^{fg}	7.13	7.00	7.07^{bcdef}
	5	7.01	7.01	7.01^{fg}	7.12	7.11	7.12^{ab}
B. japonicum	1	7.09	7.09	7.09^{abcd}	7.12	7.12	7.12^{ab}
S76	2	7.08	7.09	7.09^{abcde}	7.11	7.13	7.12^{ab}
	3	7.01	7.03	7.02^{fef}	7.11	7.01	7.06^{bcdef}
	4	7.02	7.02	7.02^{efg}	7.00	7.05	7.03^{defg}
	5	7.02	7.04	7.03^{defg}	6.98	7.00	6.99^{g}

The results as shown in Table 4.12 indicated that in the presence of 30 mM HEPES buffer, pH of YMB medium was not maintained close to the initial pH of 7.0. However, when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete alkali product(s) to increase pHs of the medium to a slightly alkali to neutral range. The same letter superscripts indicated there were no significant differences at $p < 0.05$ as determined by the Duncan's Multiple Range Test.

Table 4.13 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM HEPES buffer at the initial pH of 7.0, 30^{0}C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM HEPES			pH in YMB without 30mM HEPES		
		1	2	average	1	2	average
B. liaoningense	1	7.11	7.12	7.12	7.60	7.59	7.60
SK3	2	7.17	7.16	7.17	7.52	7.47	7.50
	3	7.12	7.13	7.13	7.63	7.58	7.61
	4	7.12	7.10	7.11	7.64	7.63	7.64
	5	7.11	7.13	7.12	7.67	7.70	7.69
B. yuanmingense	1	7.11	7.12	7.12	7.60	7.56	7.58
STB264	2	7.14	7.15	7.15	7.62	7.63	7.63
	3	7.10	7.12	7.11	7.59	7.51	7.55
	4	7.12	7.11	7.12	7.58	7.64	7.61
	5	7.12	7.14	7.13	7.69	7.68	7.69

The results as shown in Table 4.13 indicated that in the presence of 30 mM HEPES buffer, pH of YMB medium was maintained close to the initial pH of 7.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete alkali product(s) to increase pHs of the medium to a more alkali range.

Table 4.14 Responses of *B. elkanii* strain NA7 and *B. japonicum* strain S76 grown in yeast extract mannitol broth (YMB) with and without 30 mM HEPES buffer at the initial pH of 8.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM HEPES			pH in YMB without 30mM HEPES		
		1	2	average	1	2	average
B.elkanii NA7	1	8.03	8.04	8.04^{def}	8.05	8.07	8.06^{bcde}
	2	8.09	8.07	8.08^{abc}	7.99	8.00	8.00^{g}
	3	8.10	8.10	8.10^{a}	8.01	8.01	8.01^{fg}
	4	8.10	8.10	8.10^{a}	8.03	8.02	8.03^{efg}
	5	8.09	8.09	8.09^{ab}	8.09	8.11	8.10^{a}
B. japonicum	1	8.03	8.04	8.04^{def}	8.01	8.03	8.02^{fg}
S76	2	8.06	8.06	8.06^{bcde}	7.80	7.87	7.84^{j}
	3	8.03	8.06	8.05^{cdef}	7.90	7.92	7.91^{i}
	4	8.06	8.06	8.06^{bcde}	7.90	7.91	7.91^{i}
	5	8.06	8.07	8.07^{abcd}	7.93	7.97	7.95^{h}

The results as shown in Table 4.14 indicated that in the presence of 30 mM HEPES buffer, pH of YMB medium was not maintained close to the initial pH of 8.0. However, the results showed that when there was no buffer in the medium, cells of *B. elkanii* strain NA7 and *B. japonicum* strain S76 were found to secrete acidic product(s) to decrease pHs of YMB medium to a lesser alkali range. The same letter superscripts indicated there were no significant differences at $p < 0.05$ as determined by the Duncan's Multiple Range Test.

Table 4.15 Responses of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 grown in yeast extract mannitol broth (YMB) with and without 30 mM HEPES buffer at the initial pH of 8.0, 30^0C , 200 rpm, for 5 days.

Strains	Days	pH in YMB buffered with 30mM HEPES			pH in YMB without 30mM HEPES		
		1	2	average	1	2	average
B. liaoningense SK3	1	8.01	7.99	8.00^a	7.74	7.74	7.74^e
	2	8.02	8.00	8.01^a	7.83	7.76	7.80^{cd}
	3	8.01	7.99	8.00^a	7.87	7.83	7.85^{bcd}
	4	7.99	7.99	7.99^a	7.83	7.88	7.86^{bc}
	5	8.02	8.02	8.02^a	7.77	7.92	7.85^{bcd}
B. yuanmingense STB264	1	8.01	8.01	8.01^a	7.73	7.75	7.74^e
	2	8.01	8.01	8.01^a	7.87	7.87	7.87^b
	3	8.00	7.98	7.99^a	7.79	7.77	7.78^{cd}
	4	7.98	7.98	7.98^a	7.87	7.86	7.87^{bc}
	5	8.01	8.00	8.01^a	7.86	7.94	7.90^b

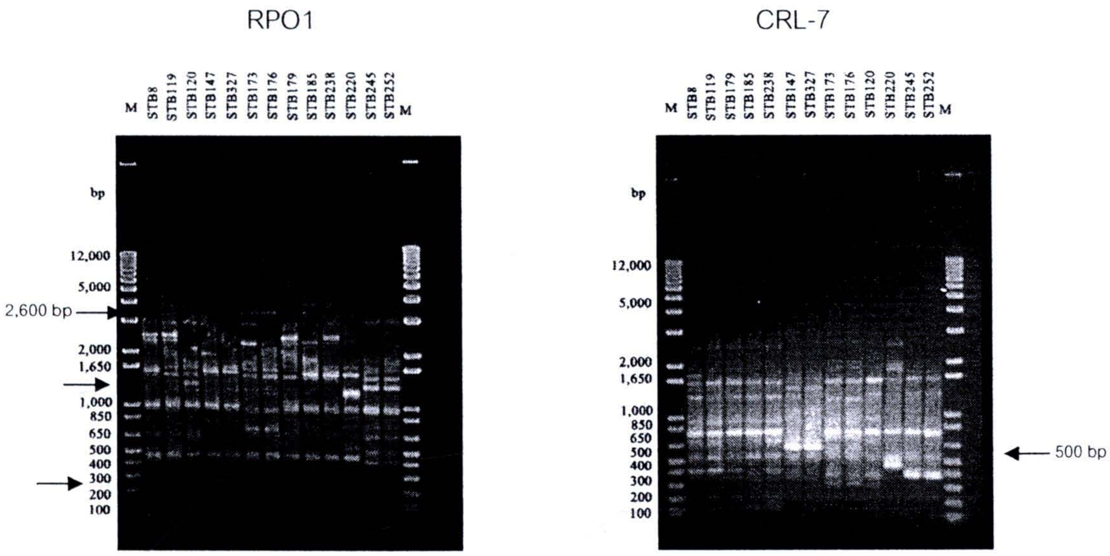
The results as shown in Table 4.15 indicated that in the presence of 30 mM HEPES buffer, pH of YMB medium was maintained close to the initial pH of 8.0. However, when there was no buffer in the medium, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete acidic product(s) to lower pHs of the medium to a lesser alkali range. The same letter superscripts indicated there were no significant differences at $p < 0.05$ as determined by the Duncan's Multiple Range Test.

The results on the maintenance of pHs when each of the three buffers was used were satisfactory in all experiments except when 30mM HEPES was used to maintain the medium pH for *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 at pH7.0 where the pHs were slightly above 7.0 as shown in Table 4.13 and when the buffer was used to maintain the medium pH for *B. elkanii* strain NA7 and *B. japonicum* strain S76 at the initial pH of 8.0 as shown in Table 4.14 where the buffered medium was slightly above pH 8.0.

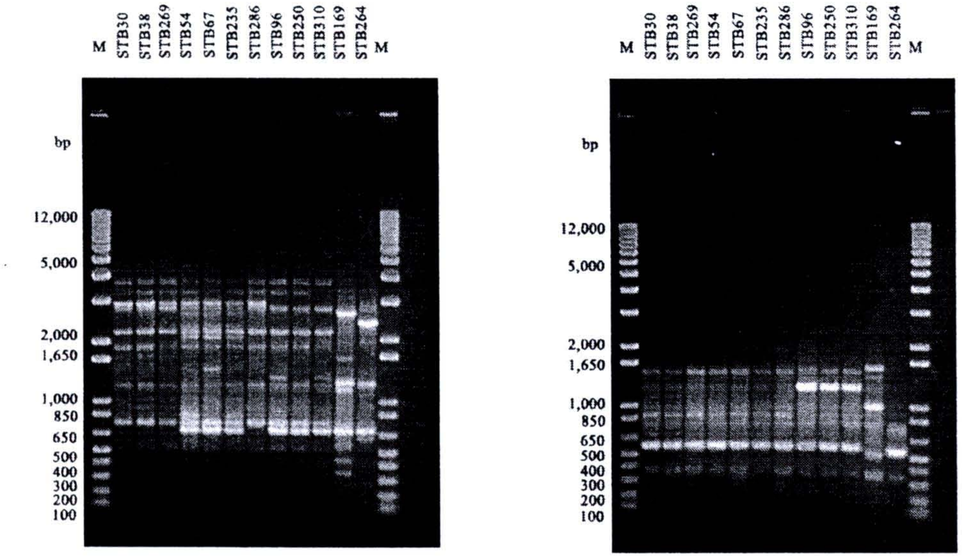
The three buffers were chosen for their effective buffering capacity as reported by Good et al. (1966). The responses of *B. elkanii* strain NA7, *B. japonicum* strain S76, *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 to changes in pHs of YMB medium when no buffer was used as shown in Tables 4.6 – 4.15 showed clearly that when pHs of the medium were in the acidic range, the slow-growing soybean rhizobium cells secreted alkali product(s) to increase the pH values to a less acidic range. On the other hand, when the medium pHs were in the neutral range, the cells were found to secrete alkali product(s) to increase the medium pHs to a slightly alkali range. However, when the medium pHs were 8.0, the cells were found to secrete acidic products to lower pHs of the medium to a lesser alkali range. Thus, the results obtained showed an ability of the slow-growing soybean rhizobia to secrete either acidic or alkali products to change pHs of the surrounding medium.

4.6.3 RAPD-PCR fingerprints of 25 randomly-selected STB slow-growing soybean rhizobium isolates

Since there was a high cost involved in the sequencing service (5,400 baht per one sequence of 16S rDNA and 1,200 baht per one sequence of *nodY*), in the use of the Transmission Electron Microscope to determine the number and type of flagella by negative staining (approximately 5,000 baht until a reportable picture of a strain was obtained), and a high cost of the Biolog^{TM} test kit (1,200 baht per one GN2 MicroPlate and inoculation fluid, and approximately 3 plates were used to obtain 3 replicates of results per strain), it was not possible to carry out polyphasic taxonomy for all the of 121 slow-growing soybean rhizobium strains. Therefore, 25 slow-growing STB isolates were randomly selected for further use in polyphasic taxonomy. In order to find out if the randomly-selected 25 STB strains were different strains, RAPD-PCR fingerprints of the 25 randomly-selected isolates were obtained as shown in Figures 4.19 (a,b).



(a)



(b)

Figure 4.19 PCR-DNA fingerprints using either RPO1 or CRL-7 as the primer of (a) 13 randomly-selected isolates and (b) 12 randomly-selected isolates. DNA fingerprints indicated the following isolates were the same strains : STB245 = STB 252 (results obtained from Figure 4.19a); STB30 = STB38 = STB269 = STB286; and STB54 = STB235 (results obtained from Figure 4.19b).

DNA fingerprints as shown in Figure 4.19 (a,b) indicated that, in fact, the 25 randomly-selected isolates consisted of 20 strains because some isolates were found to have identical DNA fingerprints so they were the same strains . Thus, the 20 strains chosen for further polyphasic taxonomy tests including 16S rDNA and *nodY* isolation and sequencing were: STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245, and STB327 (results obtained from Figure 4.19a); and STB30, STB54, STB67, STB96, STB250, STB310, STB169, STB264, (results obtained from Figure 4.19b). The observed RAPD-PCR DNA fingerprints with CRL-7 as the primer as shown in Figure 4.19 indicated less variations in the DNA fingerprint banding patterns. Further analysis of the DNA banding patterns in Figure 4.19a revealed the presence of natural variants which had near identical fingerprints except for differences either in the presence or the absence of some DNA bands. For example, RPO1 fingerprints in Figure 4.19a showed that only strains STB8, STB119, STB173 and STB185 had the 2,400 bp bands while only STB8, STB119, STB179, and STB238 had the 2,600 bp DNA bands ; only the STB strains 120 and 245 had the 1,600 bands; only STB173, STB176 and STB245 had the 700 bp DNA bands while only STB179 had the 250 bp band. Similarly, the CRL-7 fingerprints as shown in Figure 4.19a showed only the STB147 and STB327 strains had the 500 bp bands but did not have the 700 bp bands. Figure 4.19b showed that all the 6 STB strains 30, 54, 67, 96, 250, and 310 were natural variants . RPO1 fingerprints showed only STB96 and STB310 had 1,450 bp bands. In addition, only strains STB 54, 67, 96, 250, and 310 had the 700 bp bands. The CRL-7 fingerprints as shown in Figure 4.19b showed STB96, 250, and 310 had an extra 1,260 bp band.

4.6.4 Identification of slow-growing soybean rhizobia by using 16S rDNA and *nodY* sequences

4.6.4.1 Identification by 16S rDNA sequences

Table 4.16 showed identification of the selected 20 strains of slow-growing soybean rhizobia by using the Blast program to compare the obtained 16S rDNA sequences with those sequences deposited in the GenBank database. The Blast program indicated the following identification of the 20 STB strains:

STB8 (determined length 1452 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S127 . All the compared sequences had the following homology: Identities = 1451/1454 (99%), gaps = 3/1454.

STB30 (determined length 1453 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1451/1455 (99%), gaps = 4/1455.

STB54 (determined length 1451 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1446/1454 (99%), gaps = 4/1454.

STB67(determined length 1455 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1446/1454 (99%), gaps = 4/1454.

STB96 (determined length 1451 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1451/1453 (99%), gaps = 2/1415.

STB119 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S127 or *Bradyrhizobium elkanii* strain GZ1. All the compared sequences had the following homology: Identities = 1451/1452 (99%), gaps = 0/1452.

STB120 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1451 (100%), Gaps = 0/1451 or *Bradyrhizobium elkanii* strain SEMIA 5002 with identities = 1450/1452 (99%), gaps = 2/1452.

STB147 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S127or *Bradyrhizobium elkanii* strain GZ1. All the compared sequences had the following homology: Identities = 1448/1454 (99%), gaps = 5/1454.

STB169 (determined length 1449 bp) could be related to *Bradyrhizobium* sp. GX5 or *Bradyrhizobium liaoningense* strain LYG2 with identities = 1449/1454 (99%), Gaps =

4/1454 or *Bradyrhizobium yuanmingense* strain TTC4 with identities = 1447/1452 (99%), gaps = 4/1452.

STB173 (determined length 1452 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S 127 or *Bradyrhizobium elkanii* strain GZ1. All the compared sequences had the following homology: Identities = 1450/1454 (99%), gaps = 3/1454.

STB176 (determined length 1452 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 with identities = 1449/1455 (99%), gaps = 5/1455 or *Bradyrhizobium elkanii* strain SEMIA 5002 with identities = 1449/1455 (99%), gaps = 5/1455.

STB179 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S127 or *Bradyrhizobium elkanii* strain GZ1. All the compared sequences had the following homology: Identities = 1451/1451 (100%).

STB185 (determined length 1452 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 with identities = 1450/1455 (99%), gaps = 3/1455.

STB220 (determined length 1451 bp) could be related *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S 127 or *Bradyrhizobium elkanii* strain GZ1 with identities = 1448/1451 (99%) and there was no gap.

STB238 (determined length 1451 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6414 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium* *elkanii* strain S127 or *Bradyrhizobium elkanii* strain GZ1 with identities = 1450/1454 (99%), gaps = 4/1454.

STB245 (determined length 1450 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 or *Bradyrhizobium elkanii* strain SEMIA 5002 or *Bradyrhizobium elkanii* strain SEMIA 6096 or *Bradyrhizobium elkanii* strain SEMIA 6405 or *Bradyrhizobium elkanii* strain SEMIA 6416 or *Bradyrhizobium elkanii* strain S 127 or *Bradyrhizobium elkanii* strain GZ1 with identities = 1449/1454 (99%), gaps = 5/1454.

STB250 (determined length 1451 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1449/1453 (99%), gaps = 2/1453.

STB264 (determined length 1450 bp) could be related to *Bradyrhizobium* sp. GX5 or *Bradyrhizobium yuanmingense* strain TTC4 with identities = 1444/1453 (99%), gaps = 5/1453.

STB310 (determined length 1449 bp) could be related to *Bradyrhizobium japonicum* strain HMS-02 with identities = 1449/1452 (99%), gaps = 2/1452.

STB327(determined length 1454 bp) could be related to *Bradyrhizobium* sp. SEMIA 6118 with identities = 1451/1455 (99%), gaps = 3/1455 or *Bradyrhizobium elkanii* strain SEMIA 5002 with identities = 1450/1455 (99%), gaps = 5/1455.

Table 4.16 showed identification of the 20 slow-growing soybean rhizobium STB strains based on homology of 16S rDNA sequences as obtained by the Blast program of the National Center for Biotechnology Information (NCBI).

Table 4.16 Summary of identification of 20 slow-growing soybean rhizobium STB strains based on 16S rDNA sequences.

Strain	Size of 16S rDNA (bp)	Percent homology with sequences in GenBank	Identification
STB8	1452	1451/1454 (99%) with 3 gaps	*Bradyrhizobium elkanii*
STB30	1453	1451/1455 (99%) with 4 gaps	*B. japonicum*
STB54	1451	1446/1454 (99%) with 4 gaps	*B. japonicum*
STB67	1455	1449/1451 (99%) with no gap	*B. japonicum*
STB96	1451	1451/1453 (99%) with 2 gaps	*B. japonicum*

STB119	1451	1451/1452 (99%) with no gap	*B. elkanii*
STB120	1451	1450/1452 (99%) with 2 gaps	*B. elkanii*
STB147	1451	1448/1454 (99%) with 5 gaps	*B. elkanii*
STB169	1449	1449/1454 (99%) with 4 gaps 1447/1452 (99%) with 4 gaps	*B. liaoningense* *B. yuanmingense*
STB173	1452	1450/1454 (99%) with 3 gaps	*B. elkanii*
STB176	1452	1449/1455 with 5 gaps	*B. elkanii*
STB179	1451	1451/1451(100%) with no gap	*B. elkanii*
STB185	1452	1450/1455 (99%) with 3 gaps	*B. elkanii*
STB220	1451	1448/1451 (99%) with no gap	*B. elkanii*
STB238	1451	1450/1454 (99%) with 4 gaps	*B. elkanii*
STB245	1450	1449/1454 (99%) with 5 gaps	*B. elkanii*
STB250	1451	1449/1453 (99%) with gaps	*B. japonicum*
STB264	1450	1444/1453 (99%) with 5 gaps	*B. yuanmingense*
STB310	1449	1449/1452 (99%) with 2 gaps	*B. japonicum*
STB327	1454	1450/1455 (99%) with 5 gaps	*B. elkanii*

Table 4.16 indicated that the 20 STB strains consisted of 12 *B. elkanii* strains (STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245, and STB327) ; 6 *B. japonicum* strains (STB30, STB54, STB67, STB96, STB250, and STB310; one *B. yuanmingense* strain (STB264) , and one *B. liaoningense/yuanmingense* strain (STB169).

Figures 4.20 (a-c) showed dendrograms constructed with sequences of 16S rDNA of the slow-growing soybean rhizobium STB strains.

(a)

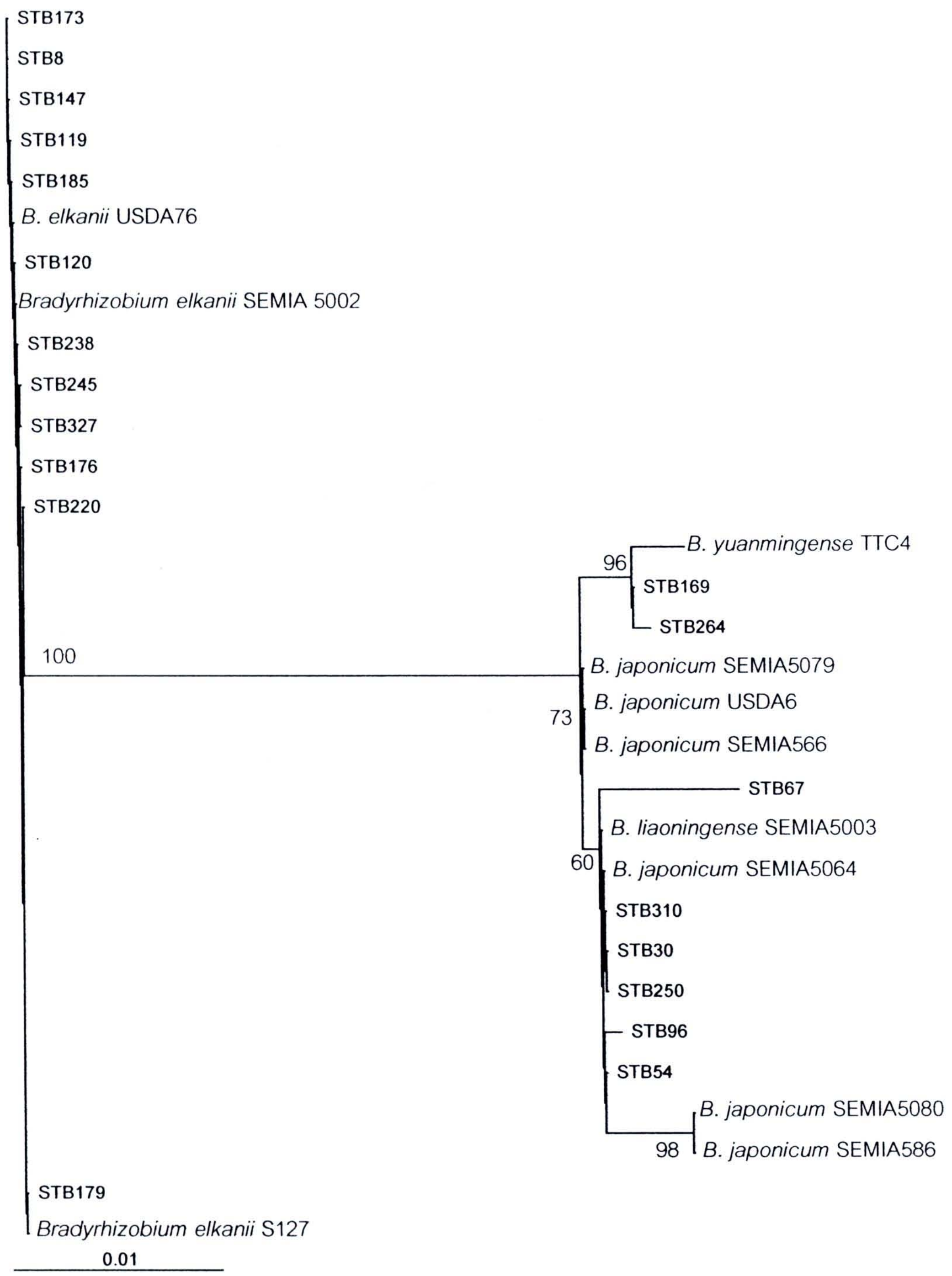


Figure 4.20a Dendrogram obtained from 16S rDNA sequences of 20 slow-growing soybean rhizobium STB strains as well as some reference strains. The Maximum Likelihood Method was used in the dendrogram construction.

(b)

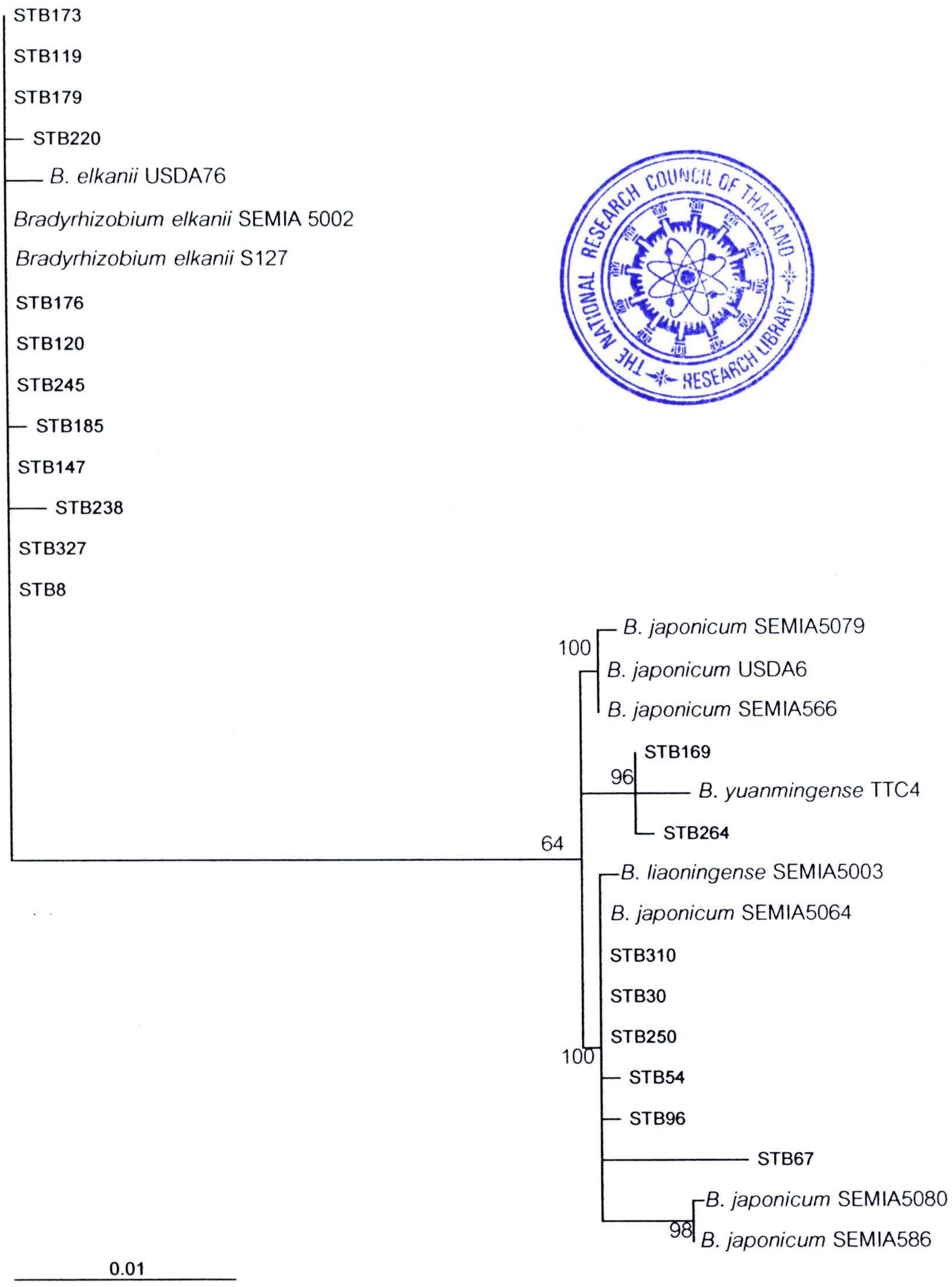


Figure 4.20b Dendrogram obtained from 16S rDNA sequences of 20 slow-growing soybean rhizobium STB strains as well as some reference strains. The Maximum Parsimony Method was used in the dendrogram construction.

(c)

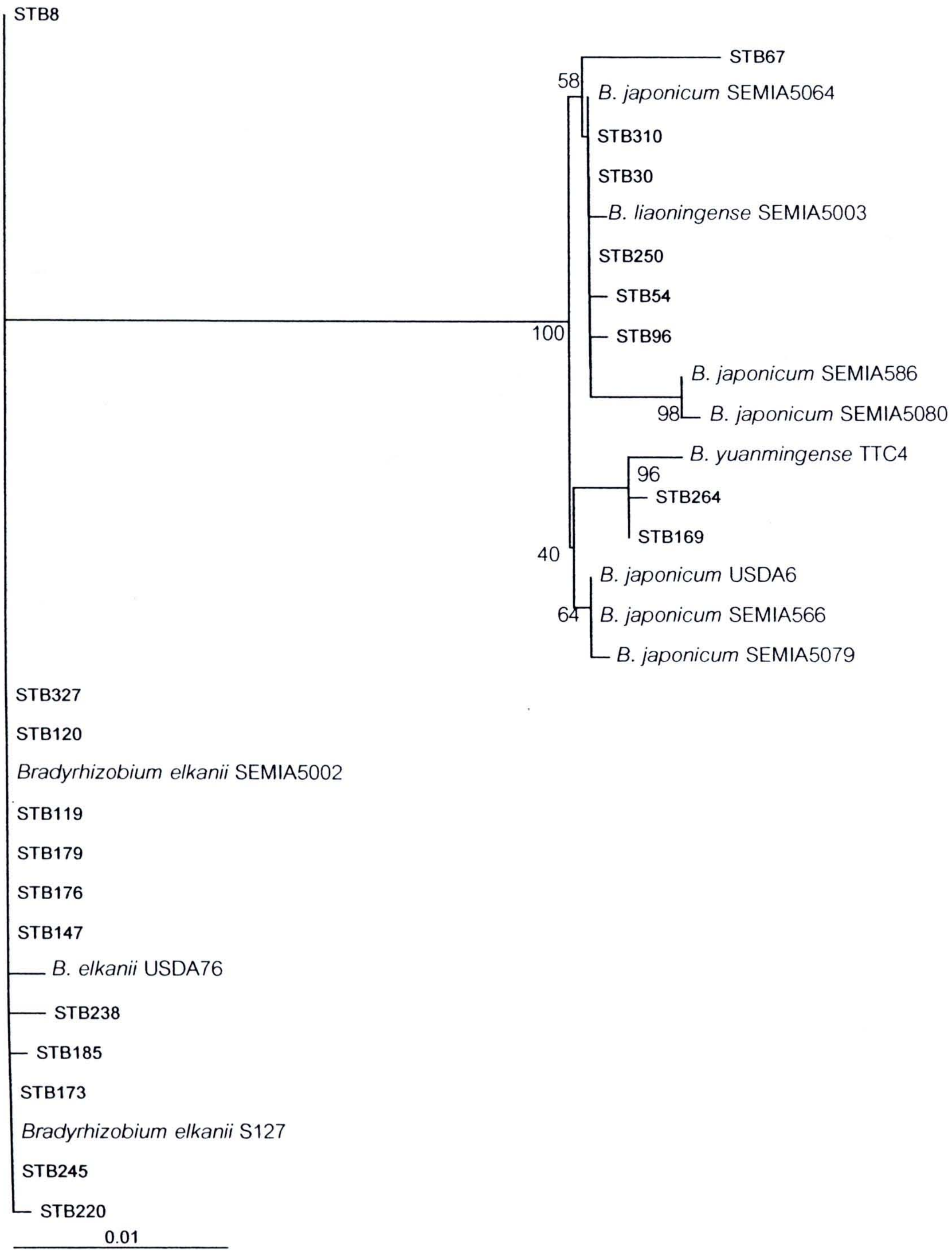


Figure 4.20c Dendrogram obtained from 16S rDNA sequences of 20 slow-growing soybean STB strains as well as some reference strains. The Neighbor-Joining Method was used in the dendrogram construction.

Figures 4.20 (a-c) showed dendrograms obtained from 16S rDNA sequences of 20 slow-growing STB strains as well as some reference strains when the Maximum Likelihood method, the Maximum Parsimony method, and the Neighbor-Joining method were used in the dendrogram construction, respectively. The results showed minor discrepancies when the three methods were used in the dendrogram construction. All the three dendrograms showed the 20 STB strains and the reference strains were grouped into 5 clusters with a minor discrepancy in Cluster I where the Maximum Likelihood dendrogram grouped the following 11 STB strains: STB173, STB8, STB147, STB119, STB185, STB120, STB238, STB245, STB327, STB176, and STB220 in the same cluster as the reference strains *B. elkanii* SEMIA 5002 and USDA 76 with STB179 grouped with *B. elkanii* S127 which had been reported as a natural variant of *B. elkanii* SEMIA 566 (Barcellos et al., 2007). However, the Maximum Parsimony method grouped STB179 into the same cluster as the 11 STB strains to yield a total of 12 STB strains of *B. elkanii* (Figure 4.20b) and the Neighbor-Joining method separated STB8 from the other STB *B. elkanii* strains (Figure 4.20c). The discrepancies observed in the three 16S rDNA dendrograms probably reflected the presence of natural variants in the *B. elkanii* STB strains.

All the three methods used in the dendrogram construction from 16S rDNA sequences revealed Cluster II consisted of the following 6 STB strains: STB310, STB30, STB250, STB96, and STB54 which were grouped with *B. japonicum* SEMIA5064/*B. liaoningense* SEMIA5003 with STB67 occupying a separate branch. The finding that *B. japonicum* SEMIA5064 and *B. liaoningense* SEMIA5003 were grouped in the same cluster probably reflected a close phylogenetic relationship between the two slow-growing soybean rhizobia species. Cluster III revealed STB169 and STB264 were closely related to *B. yuanmingense* TTC4. In addition, all the three dendrograms showed Cluster IV consisted of the reference strains *B. japonicum* SEMIA5079, USDA6, and SEMIA566. Cluster V consisted of the reference strains *B. japonicum* SEMIA5080 and SEMIA586.

4.6.4.2 Identification of slow-growing soybean rhizobia by using *nodY* sequences

Table 4.17 showed identification of the 20 slow-growing soybean rhizobium STB strains by using the Blast program to compare the obtained *nodY* sequences with those sequences in the GenBank database. The Blast program indicated the following results for *nodY* of STB strains:

STB8 (determined length 363 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 359/361 (99%), gap = 1/361 or *Bradyrhizobium elkanii* USDA94 *nodK* with Identities = 359/361 (99%), gap = 1/361.

STB30 (determined length 363 bp) could be *Bradyrhizobium* sp. TARC112 *nodY* with identities = 341/342 (99%) with 1 gap.

STB54 (determined length 364 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 362/364 (99%), gaps = 1/364.

STB67(determined length 360 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 350/353 (99%), gaps = 3/353.

STB96 (determined length 383 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 353/357 (98%), gaps = 2/357.

STB119 (determined length 354 bp) could be *Bradyrhizobium elkanii* strain USDA 76 *nodY* or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 325/329 with no gap.

STB120 (determined length 360 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 347/352 (98%), gaps = 2/352 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 347/352 (98%), gaps = 2.

STB147 (determined length 351 bp) could be *Bradyrhizobium elkanii* strain USDA 76 *nodY* with identities = 342/346 (98%), gaps = 4.

STB169 (determined length 362 bp) could be *Bradyrhizobium elkanii* USDA 31 *nodY* with identities = 329/367 (89%), gaps = 9/367.

STB173 (determined length 361 bp) could be *Bradyrhizobium elkanii* strain USDA 76 *nodY* or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 349/352, gaps = 3.

STB176 (determined length 357 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 354/361 (98%), gaps = 3/361 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 354/361 (98%), gaps = 3/361.

STB179 (determined length 359 bp) could be *Bradyrhizobium elkanii* strain USDA 76 *nodY* or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 341/345 (98%), gap = 1.

STB185 (determined length 360 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 358/361 (99%), gaps = 2/361 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 358/361 (99%), gaps = 2/361.

STB220 (determined length 359 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 329/345 (95%), gaps = 3/345 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 334/352 (94%), gaps = 3/352.

STB238 (determined length 359 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 341/353 (96%), gaps = 3/353 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 341/353 (96%), gaps = 3/353.

STB245 (determined length 358 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 328/330 (99%), gaps = 1/330 (0%) or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 326/327 (99%), gaps = 1/327.

STB250 (determined length 363 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 363/363 (100%),with no gap.

STB264 (determined length 363 bp) could be *Bradyrhizobium elkanii* USDA 31 *nodY* with identities = 363/364 (99%), gap = 1/364.

STB310 (determined length 360 bp) could be *Bradyrhizobium* sp. TARC 112 *nodY* with identities = 357/365 (97%), gaps = 6/365.

STB327(determined length 347 bp) could be *Bradyrhizobium elkanii* USDA 76 *nodY* with identities = 344/347 (99%), gaps = 1/347 or *Bradyrhizobium elkanii* USDA94 *nodK* with identities = 344/347 (99%), gaps = 1/347.

Table 4.17 showed identification of the 20 slow-growing soybean rhizobium STB strains based on homology of *nodY* sequences as obtained by the Blast program of the National Center for Biotechnology Information (NCBI).

Table 4.17 Identification of 20 slow-growing soybean rhizobium STB strains based on sequences of *nodY*.

Strains	Size of *nodY* (bp)	Percent homology with sequences in GenBank	Identification
STB8	363	359/361 (99%) with 1gap	*B.elkanii* strains USDA76, USDA94
STB30	363	341/342 (99%) with 1 gap	*Bradyrhizobium* sp. TARC112
STB54	364	362/364 (99%) with 1 gap	*Bradyrhizobium* sp. TARC112
STB67	360	350/353(99%) with 3 gaps	*Bradyrhizobium* sp. TARC112
STB96	363	353/357(98%) with 2 gaps	*Bradyrhizobium* sp. TARC112
STB119	354	325/329(98%) with no gap	*B. elkanii* strains USDA76, USDA94
STB120	360	347/352(98%) with 2 gaps	*B. elkanii* strains USDA76, USDA94
STB147	351	359/361(99%) with 1 gap	*B. elkanii* strains USDA76, USDA94
STB169	362	329/367(89%) with 9 gaps	*B. elkanii* strain USDA 31
STB173	361	349/356(98%) with 6 gaps	*B. elkanii* strains USDA76, USDA94
STB176	357	354/361(98%) with 3 gaps	strains USDA76, USDA94
STB179	359	341/345 (98%) with 1 gap	*B. elkanii* strains USDA76, USDA94
STB185	360	358/361(99%) with 2 gaps	strains USDA76, USDA94
STB220	359	329/345(95%) with 3 gaps	*B. elkanii* strains USDA76, USDA94
STB238	359	341/353(96%) with 3 gaps	*B. elkanii* strains USDA76, USDA94
STB245	358	328/330 (99%) with 1 gap	*B. elkanii* strains USDA76, USDA94
STB250	363	361/363(99%) with no gap	*Bradyrhizobium* sp. TARC112
STB264	363	363/364(99%) with 1 gap	*B. elkanii* strain USDA31
STB310	360	357/365 (97%) with 6 gap	*Bradyrhizobium* sp. TARC112
STB327	347	344/347 (99%) with 1 gap	*B. elkanii* strains USDA76, USDA94

It is interesting to note that when homology of *nodY* sequences of the 20 slow-growing soybean rhizobium STB strains and those of the strains deposited in the GenBank database were used to identify the 20 STB strains, 12 of the STB strains were found to be related to *B. elkanii* strains USDA76, USDA94, 6 STB strains were found to be related to *Bradyrhizobium* sp. TARC112, and two strains (STB 169 and STB 264) were found to be related to *B. elkanii* strain USDA31.

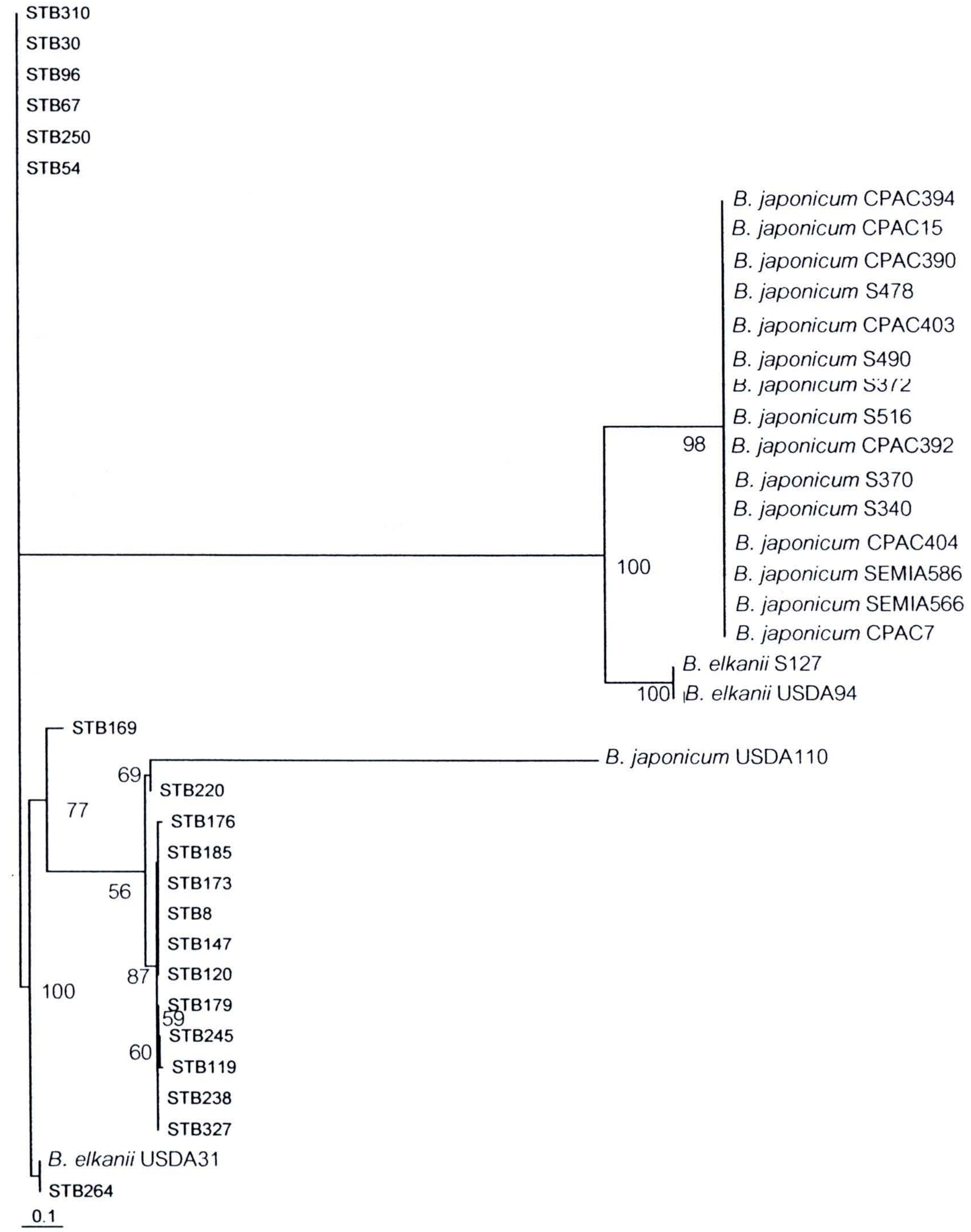


Figure 4.21a Dendrogram obtained from *nodY* sequences of 20 selected slow-growing strains as well as some reference strains. The Maximum Likelihood Method was used in the dendrogram construction.

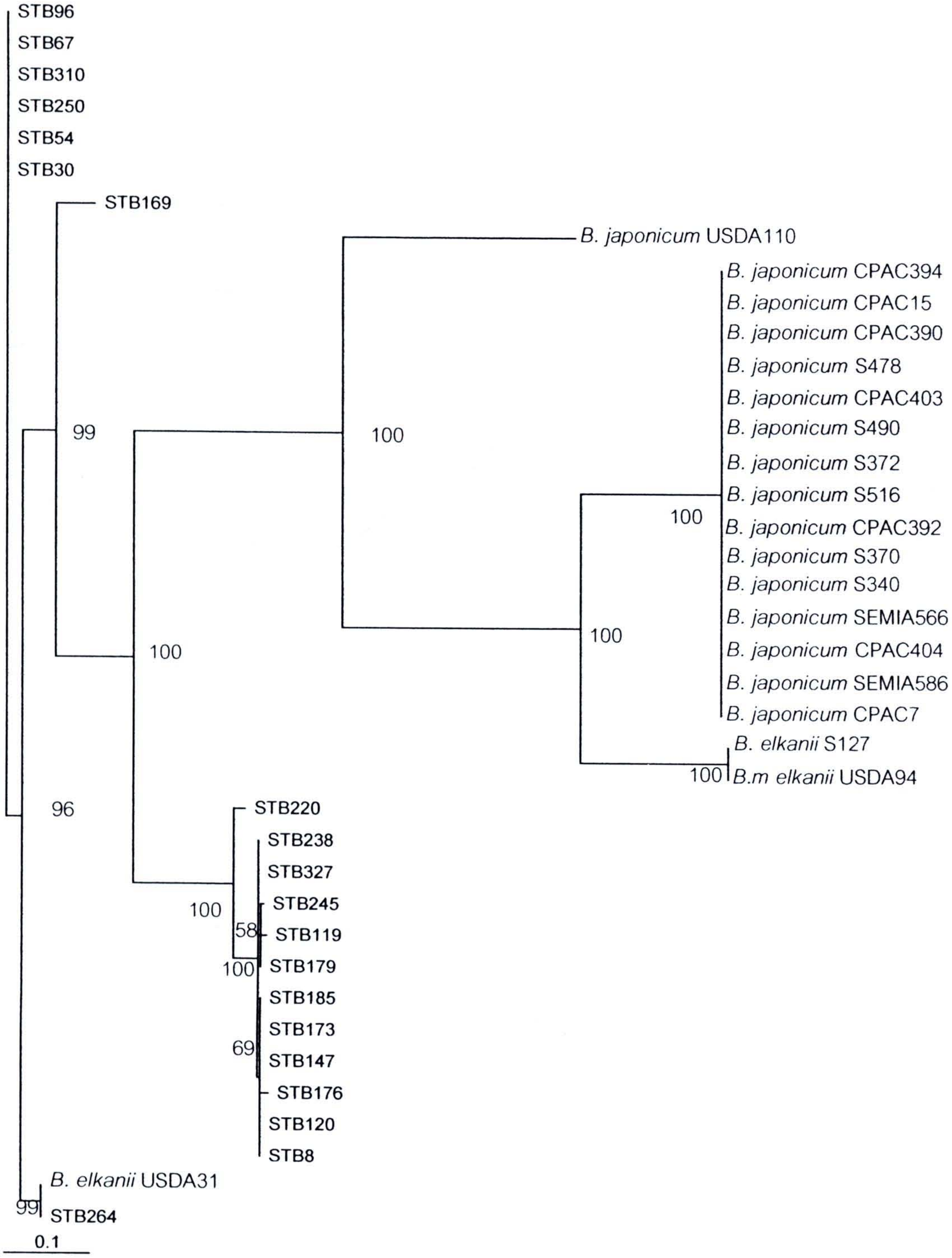


Figure 4.21b Dendrogram obtained from *nodY* sequences of 20 selected slow-growing strains as well as some reference strains. The Maximum Parsimony Method was used in the dendrogram construction.

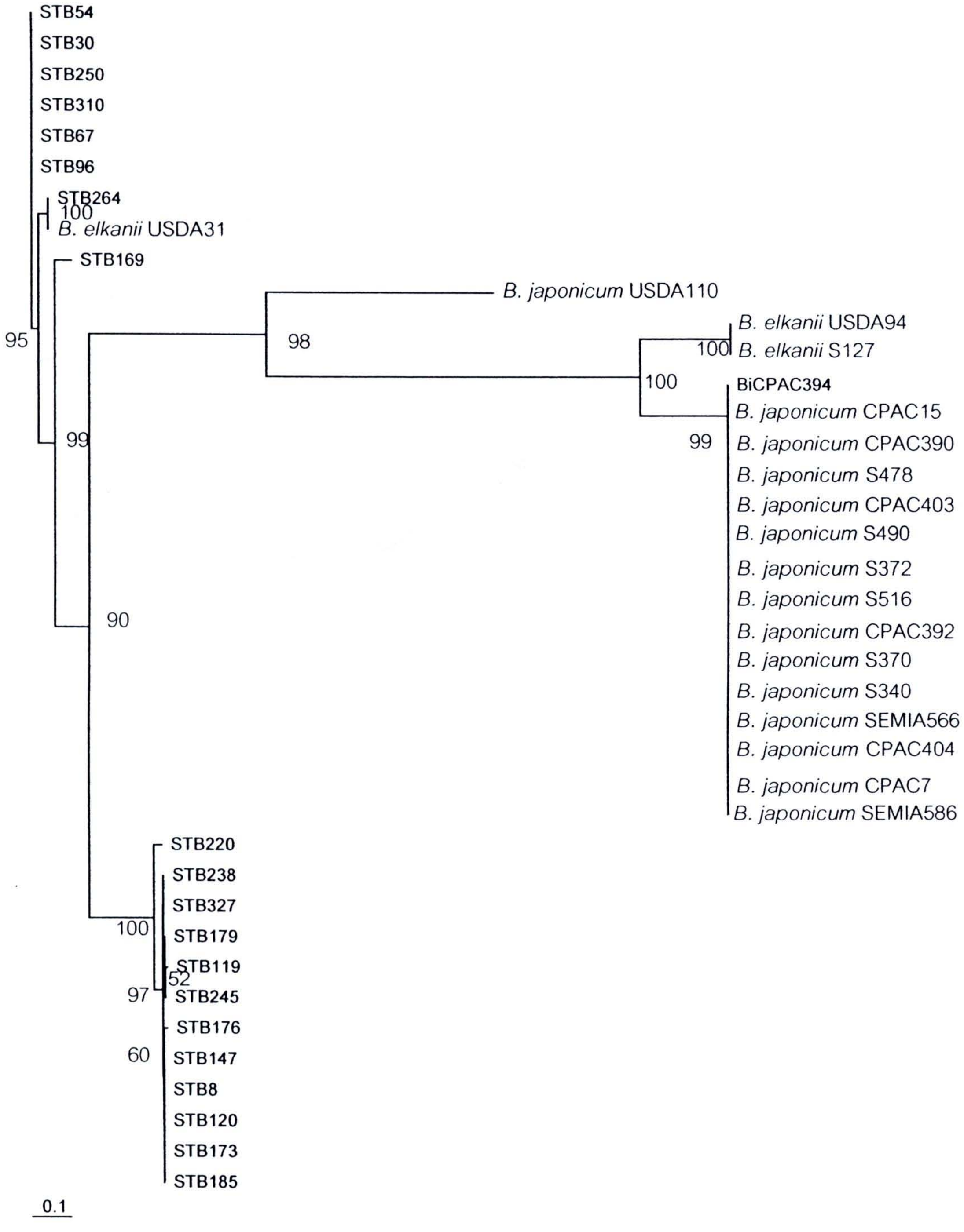


Figure 4.21c Dendrogram obtained from *nodY* sequences of 20 selected slow-growing strains as well as some reference strains. The Neighbor-Joining Method was used in the dendrogram construction.

Identification of slow-growing soybean rhizobia by using *nodY* sequences

The dendrograms constructed with *nodY* sequences of 20 STB strains and 19 reference strains as shown in Figures 4.21 (a-c) indicated a minor discrepancy when the Maximum Likelihood, Maximum Parsimony, and Neighbor-Joining methods were used in the dendrogram construction. The dendrogram constructed with the Maximum Likelihood method showed strain STB 220 as closely related to *B. japonicum* strain USDA 110 while the other methods of dendrogram construction showed the strain as closely related to all the *B. elkanii* STB strains. Apart from the discrepancy, all the three dendrograms showed the 39 strains were grouped into 5 clusters. Cluster 1 consisted of 11 or 12 *B. elkanii* STB strains (depending on whether strain STB220 was regareded as closely related to *B. elkanii* or not): STB8, 119, 120, 147, 173, 176, 179, 185, 220, 238, 245, and 327). Cluster 2 consisted of the 6 *B. japonicum* STB strains: STB 30, 54, 67, 96, 250, and 310. Cluster 3 consisted of one *B. yuanmingense* strain STB 169 which was found to be related to *B. japonicum/B. elkanii* strains S127 and USDA94 series. Cluster 4 showed *B. yuanmingense* strain STB264 as related to *B.elkanii* strain USDA 31. Cluster 5 contained the rest of the 18 reference strains. The results showed the same phylogenetic relationships as obtained with the 16S rDNA dendrograms except for the fact that STB strains 169 and 264 were not shown to be closely related to *B. yuanmingense* because there were no *nodY* sequences of *B. yuanmingense* deposited in the GenBank database for downloading.

Since 16S rDNA sequences are relatively longer (1500 bp) than *nodY* sequences (approximately 350 bp) and there are not many sequences of *nodY* deposited in the Genbank database, the identification and phylogenetic relationship determination of slow-growing soybean rhizobia were more reliable when 16S rDNA sequences were used in the identification and phylogenetic relationship determination. Therefore, the 20 slow-growing soybean rhizobium strains were identified as 12 *B. elkanii* strains, 6 *B. japonicum* strains, and 2 *B. yuanmingense* strains. This research provided the first record of the presence of *B. yuanmingense* in Thailand. Appendix D showed 16S rDNA and *nodY* sequences of 20 slow-growing soybean rhizobium STB strains. Figure 4.22 showed the distribution of the 20 STB strains according to the isolation sites of 16 subdistricts in Phitsanulok province.

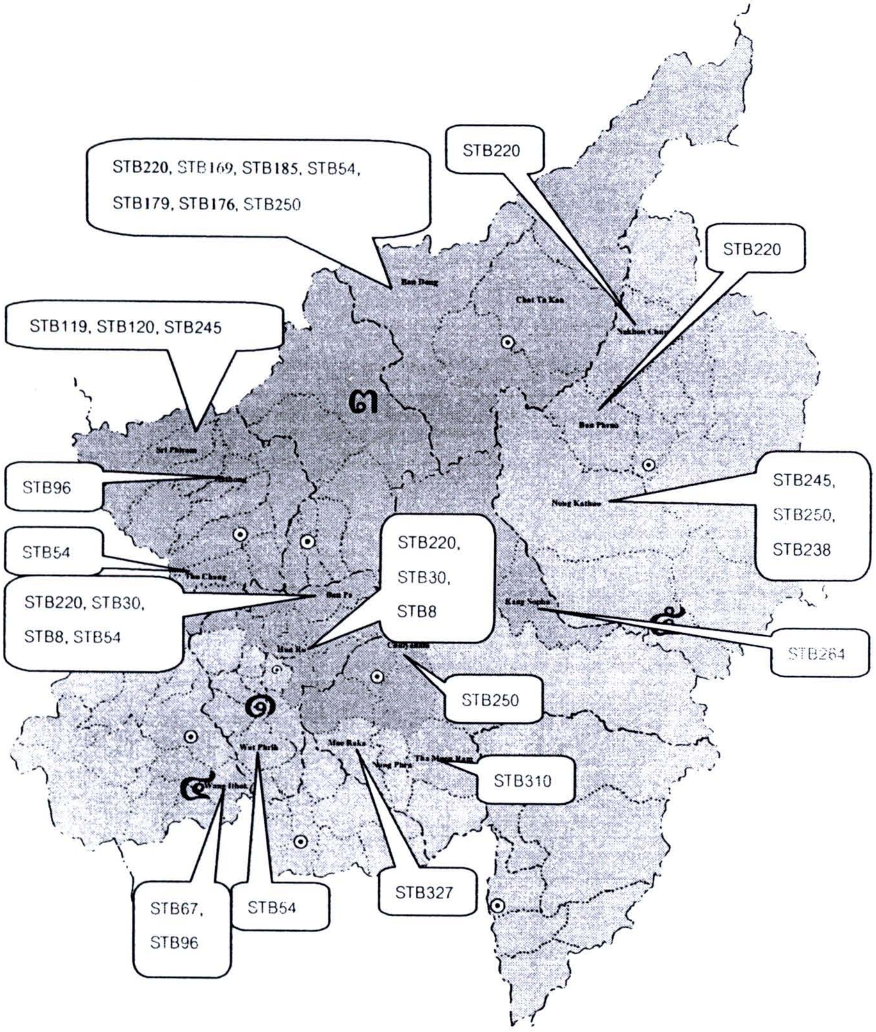
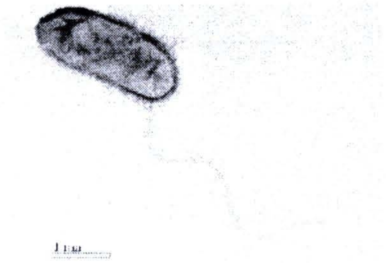


Figure 4.22 Distribution of 12 *Bradyrhizobium elkanii* STB strains (STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB238, STB245, and STB327), 6 *Bradyrhizobium japonicum* STB strains (STB30, STB54, STB67, STB96, STB250 and STB310), and 2 *Bradyrhizobium yuanmingense* (STB169 and STB264) in 16 subdistricts in Phitsanulok province, Thailand.

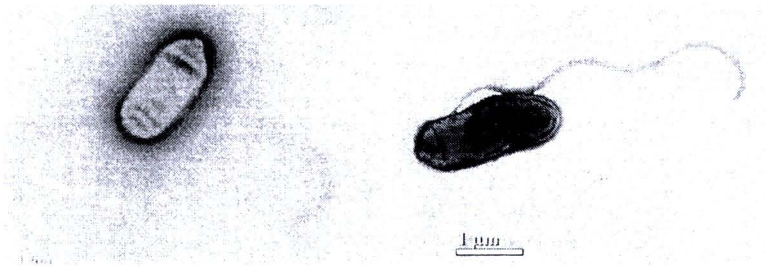
The distribution of the 20 slow-growing soybean rhizobium STB strains as shown in Figure 4.22 showed the presence of *B. elkanii* in almost all of the 16 subdistricts.

4.6.5 Number and type of flagella as determined by negative staining

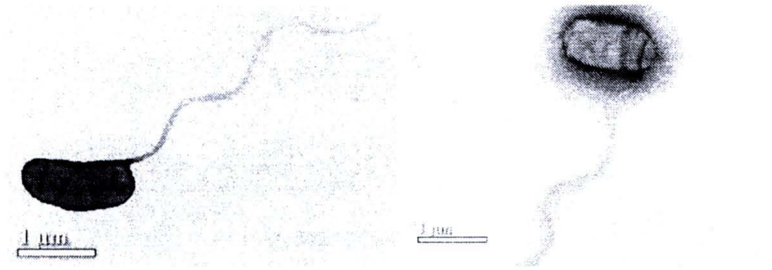
Five strains of slow-growing soybean rhizobia were selected for negative staining of flagella. The results in Figure 4.23 showed each of the 5 selected strains had one sub-polar flagellum as expected. Elkan and Bunn (1992) reported slow-growing soybean rhizobia had one sub-polar flagellum. The length of flagella may play a role in the competitive ability of soybean rhizobia in the root nodulation process (Vlassak and Vanderleyden, 1997). However, other factors including soybean cultivars also play a role in the competitive ability of soybean rhizobia in the root nodulation process (Payakapong et al., 2004).



B. japonicum STB 30



B. japonicum STB96 *B. elkanii* STB120

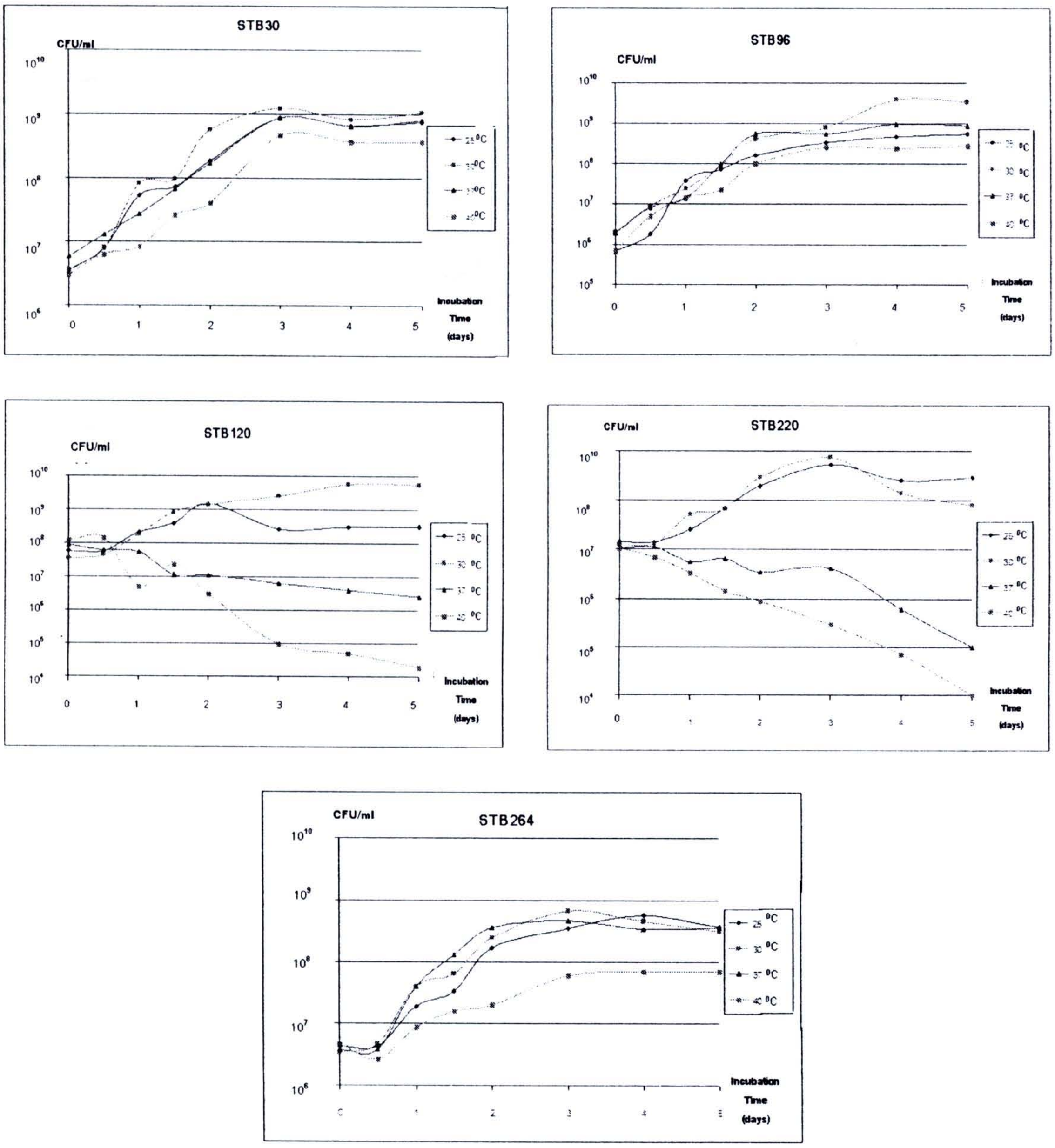


B. elkanii STB220 *B. yuanmingense* STB264

Figure 4.23 Transmission electron micrographs of 5 slow-growing soybean rhizobium STB strains.

4.6.6 Determination of growth at different temperatures

Figures 4.24 showed growth at different temperatures in terms of CFU/ml of 5 selected strains of isolated slow-growing soybean rhizobia. Table 4.18 showed the calculated specific growth rates.



Figures 4.24 Growth at different temperatures in terms of CFU/ml of 5 selected strains of isolated slow-growing soybean rhizo

Table 4.18 Specific growth rates of 5 slow-growing STB strains.

Strains	Specific growth rates at different temperatures (days^{-1})			
	25°C	30°C	37°C	40°C
B. japonicum STB 30	1.99	2.54	1.69	1.26
B. japonicum STB 96	2.07	1.74	1.89	1.99
B. elkanii STB 120	1.53	1.13	Cells died	Cells died
B. elkanii STB 220	1.21	1.36	Cells died	Cells died
B.yuanmingense STB264	1.83	2.14	2.30	0.74

The growth and specific growth rates of the 5 selected STB strains as shown in Figure 4.24 and Table 4.18 indicated the *B. japonicum* and the *B.yuanmingense* strains grew better at the relatively low temperatures at 25°C and 30°C. The *B. elkanii* strains were found to be heat-sensitive. The results showed different intrinsic abilities of the slow-growing soybean rhizobia to grow at different temperatures.

4.6.7 Ability/Inability to utilize different kinds of carbon and nitrogen sources

Table 4.19 showed results on the ability/inability of the 20 slow-growing soybean rhizobium STB strains and three reference strains of slow-growing soybean rhizobia to use different kinds of carbon and nitrogen sources as determined by the Biolog™ test kit.

Table 4.19 Ability/Inability to utilize 95 carbon and nitrogen sources as determined by the Biolog™ test kit of the 20 slow-growing soybean rhizobium STB strains and three reference strains. + indicates the strains could use the carbon and nitrogen sources. – indicates the strains could not use the carbon and nitrogen sources. Numbers indicate the total numbers out of the 20 test strains that could use the carbon and nitrogen sources.

Carbon/Nitrogen sources	*B. elkanii* NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	Numbers of strains
α-Cyclodextrin	-	-	-	4
Dextrin	+	+	+	15

Glycogen	-	-	+	1
Tween 40	+	+	+	20
Tween 80	+	+	+	20
N-Acetyl-D-Galactosamine	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-
Adonitol	+	-	-	2
L-Arabinose	+	+	+	20
D-Arabitol	+	+	-	18
D-Cellobiose	-	+	-	2
i-Erythritol	-	-	-	-
D-Fructose	+	+	-	16
L-Fucose	+	-	-	10
D-Galactose	+	+	+	20
Gentiobiose	-	-	-	-
α-D-Glucose	+	+	+	20
m-Inositol	-	-	-	-
α-D-Lactose	-	-	-	-
Lactulose	-	-	-	-
Maltose	-	-	-	-
D-Mannitol	+	+	+	20
D-Mannose	+	+	+	20
D-Melibiose	-	-	-	-
β-Methyl-D-Glucoside	+	-	-	-
D-Psicose	+	-	-	9
D-Raffinose	-	-	-	-
L-Rhamnose	-	+	-	8
D-Sorbitol	+	-	-	9
Sucrose	-	-	-	-
D-Trehalose	-	-	-	-
Turanose	-	-	-	-

Xylitol	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	20
Succinic Acid Mono-Methyl-Ester	+	+	+	20
Acetic Acid	+	+	+	20
Cis-Aconitic Acid	-	-	-	-
Citric Acid	+	-	-	9
Formic Acid	+	+	-	15
D-Galactonic Acid Lactone	+	+	-	15
D-Galacturonic Acid	+	-	-	11
D-Gluconic Acid	+	+	+	20
D-Glucosaminic Acid	+	-	-	11
D-Glucuronic Acid	+	-	-	2
α-Hydroxybutyric Acid	-	+	+	8
β-Hydroxybutyric Acid	+	+	+	20
γ-Hydroxybutyric Acid	+	+	+	20
p-Hydroxy Phenylacetic Acid	+	-	+	3
Itaconic acid	-	+	+	8
α-Keto Butyric Acid	-	+	+	5
α-Keto Glutaric Acid	+	+	+	15
α-Keto Valeric Acid	-	+	+	11
D,L-Lactic Acid	+	+	+	20
Malonic Acid	+	-	+	3
Propionic Acid	+	+	+	20
Quinic Acid	+	-	-	2
D-Saccharic Acid	+	+	+	18
Sebacic Acid	+	-	+	14
Succinic Acid	+	+	+	20
Bromosuccinic Acid	+	+	+	20
Succinamic Acid	+	+	+	20
Glucuronamide	+	-	-	7

L-Alaninamide	+	+	-	14
D-Alanine	+	-	-	11
L-Alanine	+	-	+	5
L-Alanyl-glycine	+	+	-	3
L-Asparagine	+	-	-	8
L-Aspartic Acid	+	-	+	11
L-Glutamic Acid	+	-	-	12
Glycyl-L-Aspartic Acid	+	-	-	3
Glycyl-L-Glutamic Acid	-	-	+	5
L-Histidine	-	-	-	-
Hydroxy-L-Proline	-	-	-	-
L-Leucine	+	+	+	20
L-Ornithine	-	-	+	1
L-Phenylalanine	+	+	+	20
L-Proline	+	+	-	13
L-Pyroglutamic Acid	+	+	+	18
D-Serine	+	-	-	10
L-Serine	+	-	-	2
L-Threonine	-	+	-	5
D,L-Carnitine	-	-	-	-
γ-Amino Butyric Acid	-	+	-	5
Urocanic Acid	-	-	-	-
Inosine	-	-	-	-
Uridine	-	-	-	-
Thymidine	-	-	-	-
Phenyethyl-amine	-	-	-	-
Putrescine	-	-	-	-
2-Aminoethanol	-	-	-	-
2,3-Butanediol	+	-	-	1
Glycerol	+	+	+	18

D,L-α-Glycerol Phosphate	+	-	-	3
α-D-Glucose-1-Phosphate	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-

The results indicated all the 20 slow-growing soybean rhizobium STB strains could utilize Tween 40, Tween 80, L-Arabinose , D-Galactose, α-D-Glucose, D-Mannitol, D-Mannose , Pyruvic Acid Methyl Ester, Succinic Acid Mono-Methyl-Ester, Acetic Acid, D-Gluconic Acid, β-Hydroxybutyric Acid , γ-Hydroxybutyric Acid, D,L-Lactic Acid, Propionic Acid, Succinic Acid , Bromosuccinic Acid , Succinamic Acid , L-Leucine , and L-Phenylalanine. The 20 slow-growing soybean rhizobium STB strains could not utilize many carbon and nitrogen sources as shown in Table 4.19. The results indicated that there were variations in the ability to use different kinds of carbon and nitrogen sources even within the strains that were identified as belonging to the same genus and species. The results confirmed the existence of genetic variations as observed in the different RAPD-PCR fingerprints of strains belonging to the same genera and species. Appendix E showed a summary of representative results of ability/inability to use 95 carbon/nitrogen sources by three reference strains and by *B. elkanii* strain STB327, *B. japonicum* strain STB310 and *B. yuanmingense* strain STB264.

4.7 Genetic diversity of 121 slow-growing soybean rhizobium STB strains

Figure 4.25 (a,b) showed dendrograms constructed from DNA fingerprints of 121 slow-growing soybean rhizobium STB strains when either RPO1 or CRL-7 was used as the primer. The dendrograms showed two main groups of slow-growing soybean rhizobia as follows:

Group 1 consisted of 2 subgroups : Subgroups 1.1 and 1.2.

Subgroup 1.1 contained three clusters :

Cluster 1.1.1 consisted of 2 subclusters, the first subcluster 1.1.1.1 consisted of 11 STB strains (STB209, STB220, STB226 , STB236 , STB231 , STB234 , STB233 , STB222 , STB214 , STB224 ,and STB213) which were found to be closely related to *B. elkanii* STB220. The second subcluster 1.1.1.2 consisted of 12 strains (STB171 , STB173 , STB179 , STB150 , STB162 , STB142 , STB168 , STB169 , STB147 , STB178 , STB154 , and STB163) which were found to be closely related to *B. elkanii*

STB147, *B. elkanii* STB173, *B. elkanii* STB176, and *B. elkanii* STB179/ *B. yuanmingense* STB169.

Cluster 1.1.2 consisted of 2 subclusters: subcluster 1.1.2.1 consisted of 7 strains (STB116, STB117, STB100, STB113, STB96, STB114, and STB89) which were found to be closely related to *B. japonicum* STB96. Subcluster 1.1.2.2 contains 16 strains (STB295, STB299, STB288, STB328, STB281, STB282, STB292, STB327, STB328, STB330, STB294, STB298, STB284, STB286, STB285, and STB296) which were found to be closely related to *B. elkanii* STB327.

Cluster 1.1.3 consisted of 12 strains (STB261, STB270, STB268, STB271, STB274, STB276, STB259, STB256, STB266, STB264, STB273, and STB275) which were found to be closely related to *B. yuanmingense* STB264.

Subgroup 1.2 consisted of 2 clusters:

Cluster 1.2.1 consisted of 2 subclusters. The first subcluster 1.2.1.1 consisted of 8 strains (STB250, STB255, STB248, STB243, STB254, STB237, STB238, and STB252) which were found to be closely related to *B. elkanii* STB238/*B. japonicum* STB250. The second subcluster 1.2.1.2 consisted of 4 strains (STB325 , STB326 , STB245 , and STB241) which were found to be closely related to *B. elkanii* STB245. The third subcluster 1.2.1.3 consisted of 9 strains (STB333, STB335, STB332, STB318, STB319, STB300, STB336, STB302, and STB310) which were found to be closely related to *B. japonicum* STB310.

Cluster 1.2.2 consisted of 4 strains (STB46, STB56, STB67, and STB63) which were found to be closely related to *B. japonicum* STB54 and *B. japonicum* STB67.

Group 2 consisted of 2 subgroups: subgroup 2.1 and subgroup 2.2. Subgroup 2.1 was divided into three clusters:

Cluster 2.1.1 consisted of 12 strains (STB321, STB340, STB131, STB120, STB121, STB126, STB132, STB28, STB8, STB3, STB1 and STB201) which were found to be closely related to *B. elkanii* STB8 and *B. elkanii* STB120.

Cluster 2.1.2 consisted of 10 strains (STB180, STB320, STB189, STB203, STB202, STB183, STB185, STB119, STB181 and STB139) which were found to be closely related to *B. elkanii* STB185 and *B. elkanii* STB119.

Cluster 2.1.3 consisted of 4 strains (STB188, STB41, STB38, and STB4) which were found to be closely related to *B. japonicum* STB30.

Subgroup 2.2. contained 12 strains whose identities could not be obtained from the experimental results because no previously-identified soybean rhizobia were grouped in the same subgroup 2.2.

Dendrograms obtained from RPO1 and CRL-7 DNA fingerprints showed identical clustering of the 121 slow-growing soybean rhizobia strains used in this research. The strains were divided into two groups, namely, Groups 1 and 2. Group 1 was divided into two subgroups. Subgroup 1.1 contained 3 clusters. Cluster 1.1.1 contained two subclusters 1.1.1.1 and 1.1.1.2. Cluster 1.1.2 contained two subclusters 1.1.2.1 and 1.1.2.2. Cluster 1.1.3 formed one cluster. Subgroup 1.2 contained two clusters. Cluster 1.2.1 contained three subclusters 1.2.1.1, 1.2.1.2, and 1.2.1.3. Cluster 1.2.2 formed one cluster. Group 2 contained two subgroups. Subgroup 2.1 contained three clusters 2.1.1, 2.1.2, and 2.1.3. Subgroup 2.2 contained one cluster. The clustering was summarized in Figure 4.25(a). The results as shown in Figure 4.25(b) showed 73 strains of slow-growing soybean rhizobia constituted seven types of strains which were found to be closely related to *B. elkanii*. The first type consisted of 11 strains in subcluster 1.1.1.1 which were found to be closely related to *B. elkanii* STB220. The second type contained 12 strains in subcluster 1.1.1.2 which were found to be closely related to *B. elkanii* STB147, *B. elkanii* STB173, *B. elkanii* STB176, *B. elkanii* STB179/ *B. yuanmingense* STB169. The third type of slow-growing soybean rhizobia contained 16 strains in subcluster 1.1.2.2 which were found to be closely related to *B. elkanii* STB327. The fourth type of slow-growing soybean rhizobia consisted of 8 strains in subcluster 1.2.1.1 which were found to be closely related to *B. elkanii* STB328 / *B. japonicum* STB250. The fifth type of slow-growing soybean rhizobia consisted of 4 strains in subcluster 1.2.1.2 which were found to be closely related to *B. elkanii* STB245. The sixth type of slow-growing soybean rhizobia consisted of 12 strains in cluster 2.1.1 which were found to be closely related to *B. elkanii* STB8 and *B. elkanii* STB120. The seventh type of slow-growing soybean rhizobia consisted of 10 strains in cluster 2.1.2

which were found to be closely related to *B. elkanii* STB185 and *B. elkanii* STB119. In addition, 24 strains of isolated slow-growing soybean rhizobia in subclusters 1.1.2.1, 1.2.1.3 and in clusers 1.2.2, and 2.1.3 were found to be closely related to *B. japonicum* STB96, *B. japonicum* STB310, and *B. japonicum* STB54, *B. japonicum* STB67, and *B. japonicum* STB30 repectively. Moreover, 12 strains of slow-growing soybean rhizobia in cluster 1.1.3 were found to be closely related to *B. yuanmingense* STB264. With the twelve strains of unidentified slow-growing soybean rhizobia, the total number of slow-growing soybean rhizobia was 121 strains consisting of 73 *B. elkanii* strains, 24 *B. japonicum* strains, 12 *B. yuanmingense* strains and 12 unidentified strains.

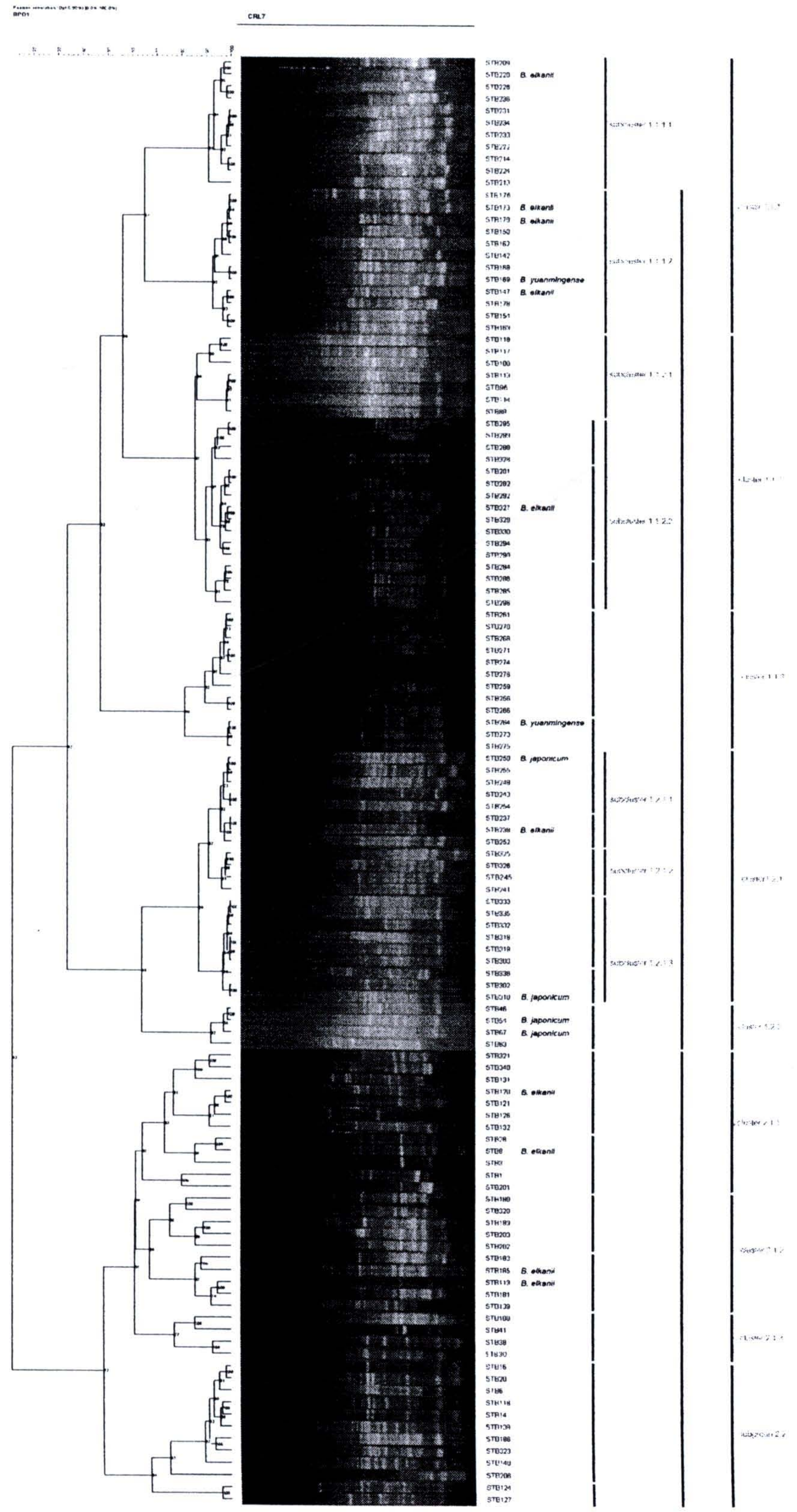


Figure 4.25 (a) Dendrogram constructed from DNA fingerprints using CRL-7 as the primer. Detail is given in text.

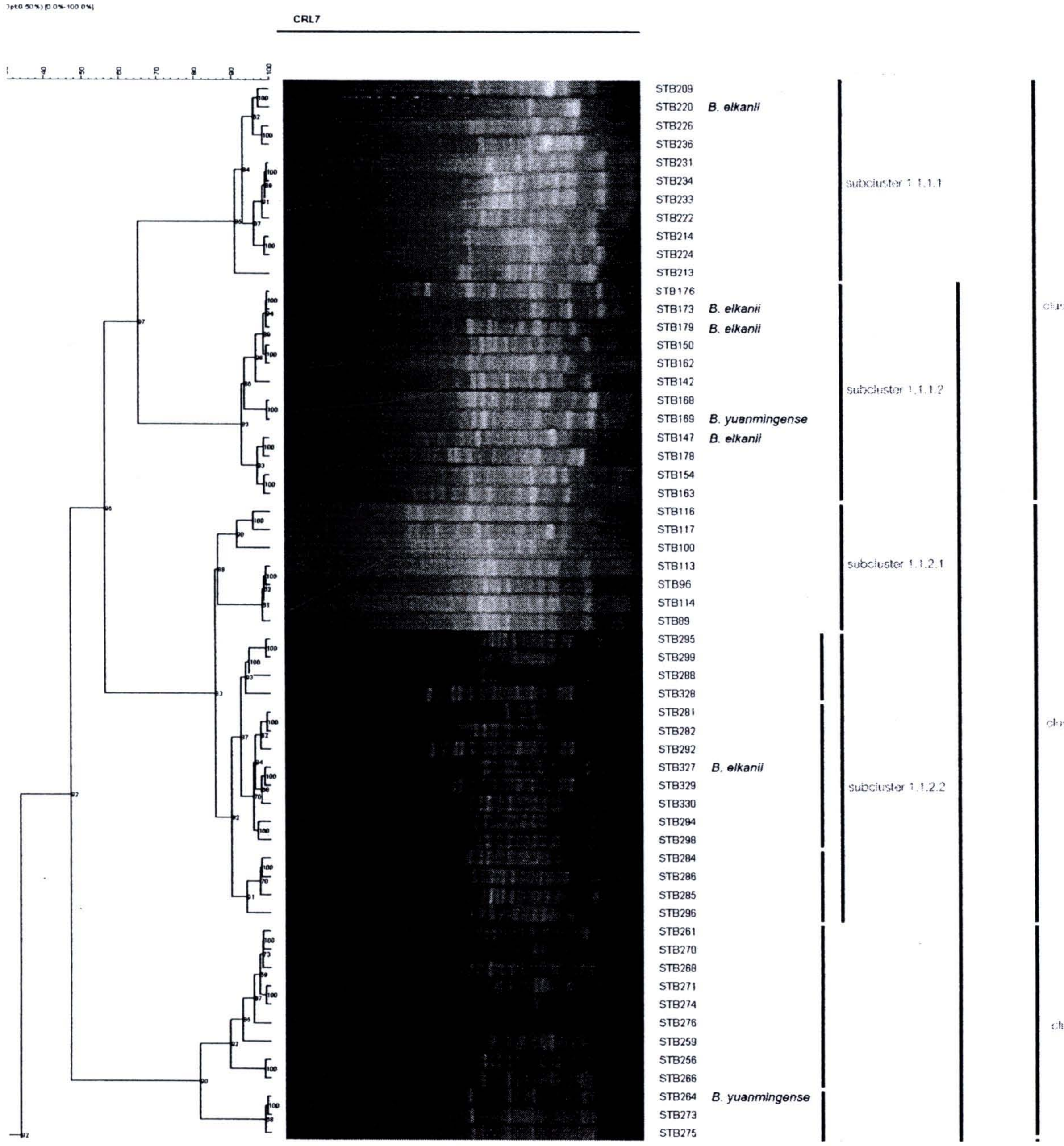


Figure 4.25 (a) Dendrogram constructed from DNA fingerprints using CRL-7 as the primer. Detail is given in text.

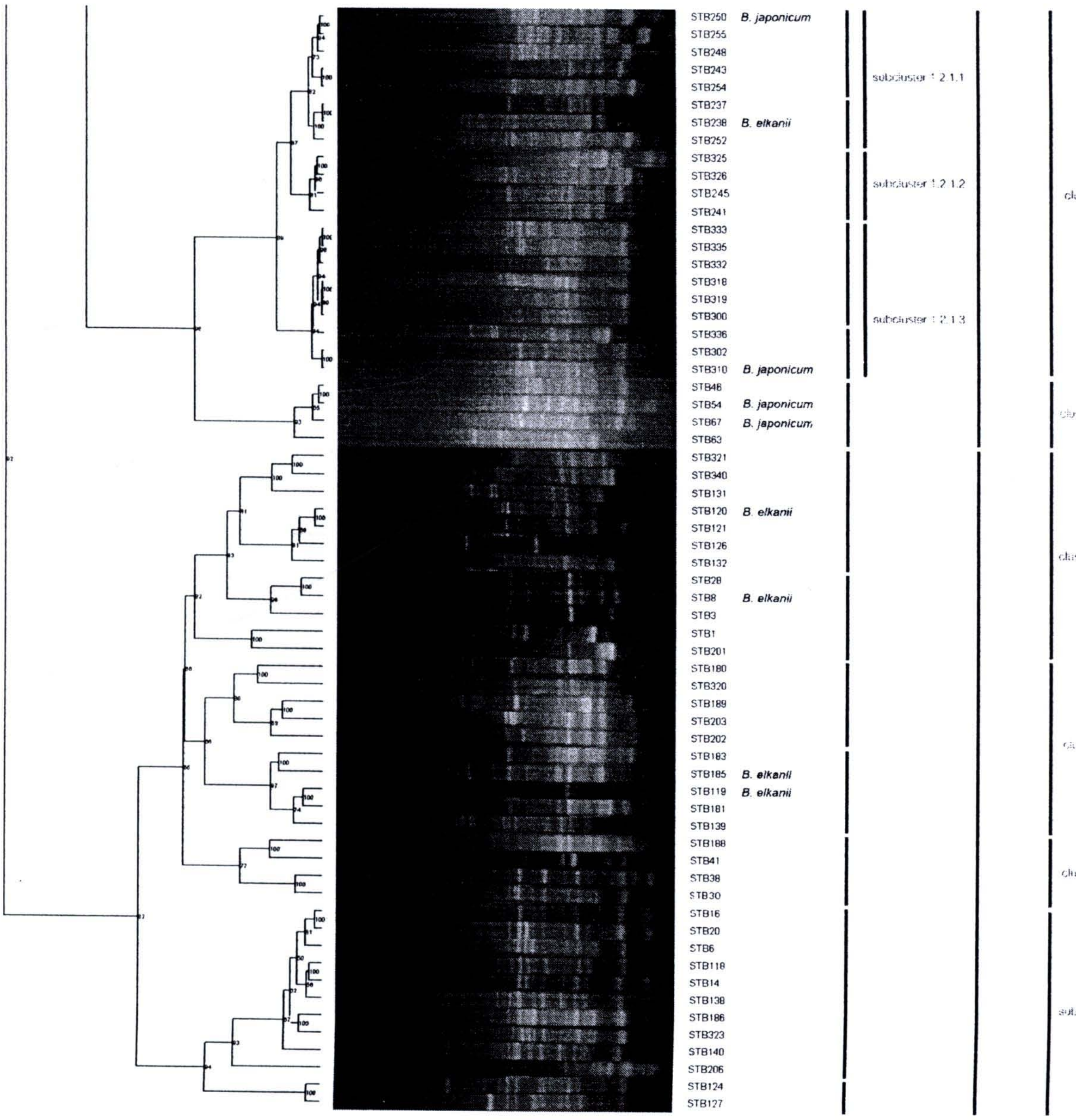


Figure 4.25 (a) Dendrogram constructed from DNA fingerprints using CRL-7 as the primer. Detail is given in text.

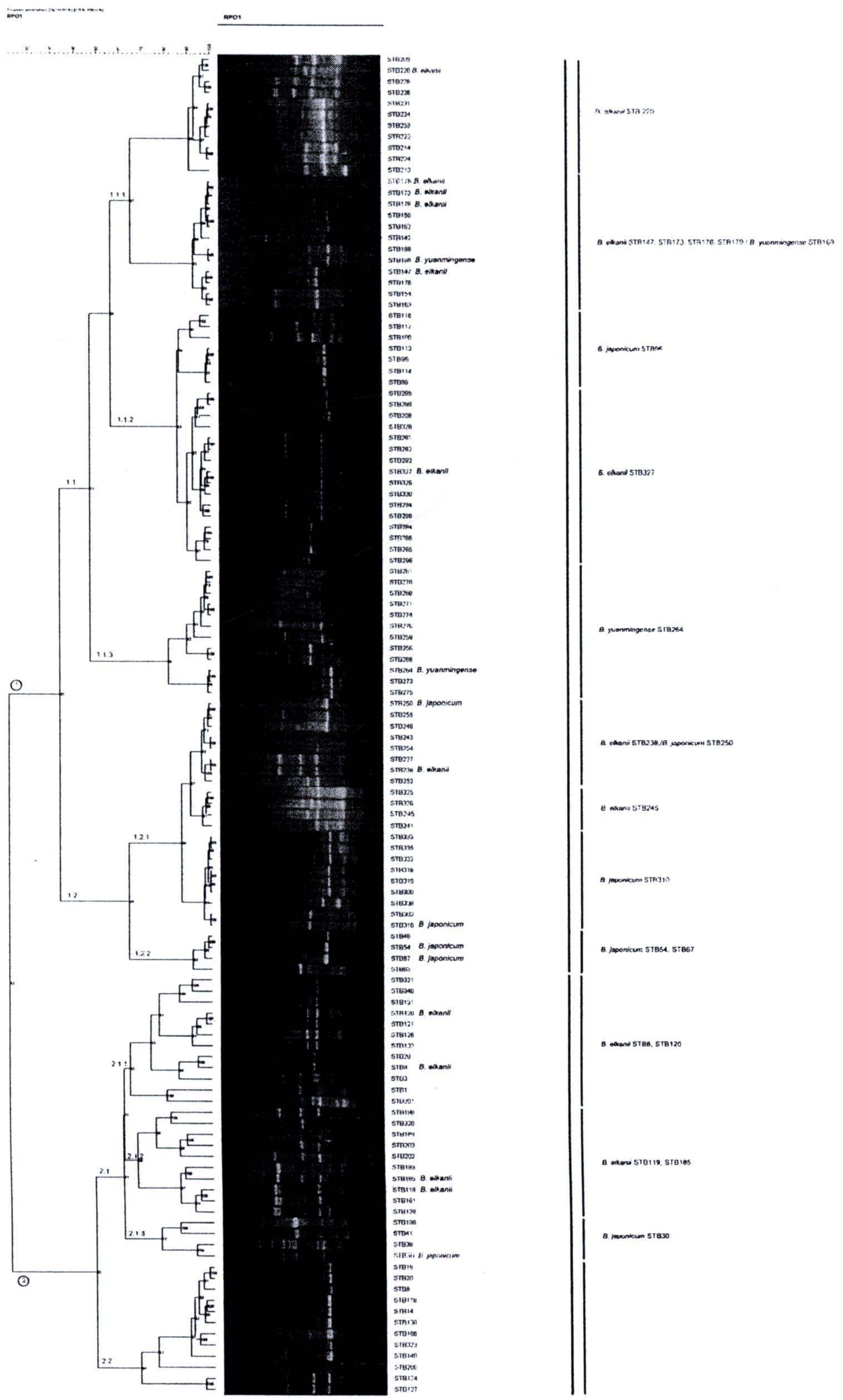


Figure 4.25 (b) Dendrogram constructed from DNA fingerprints using RPO1 as the primer. Detail is given in text.

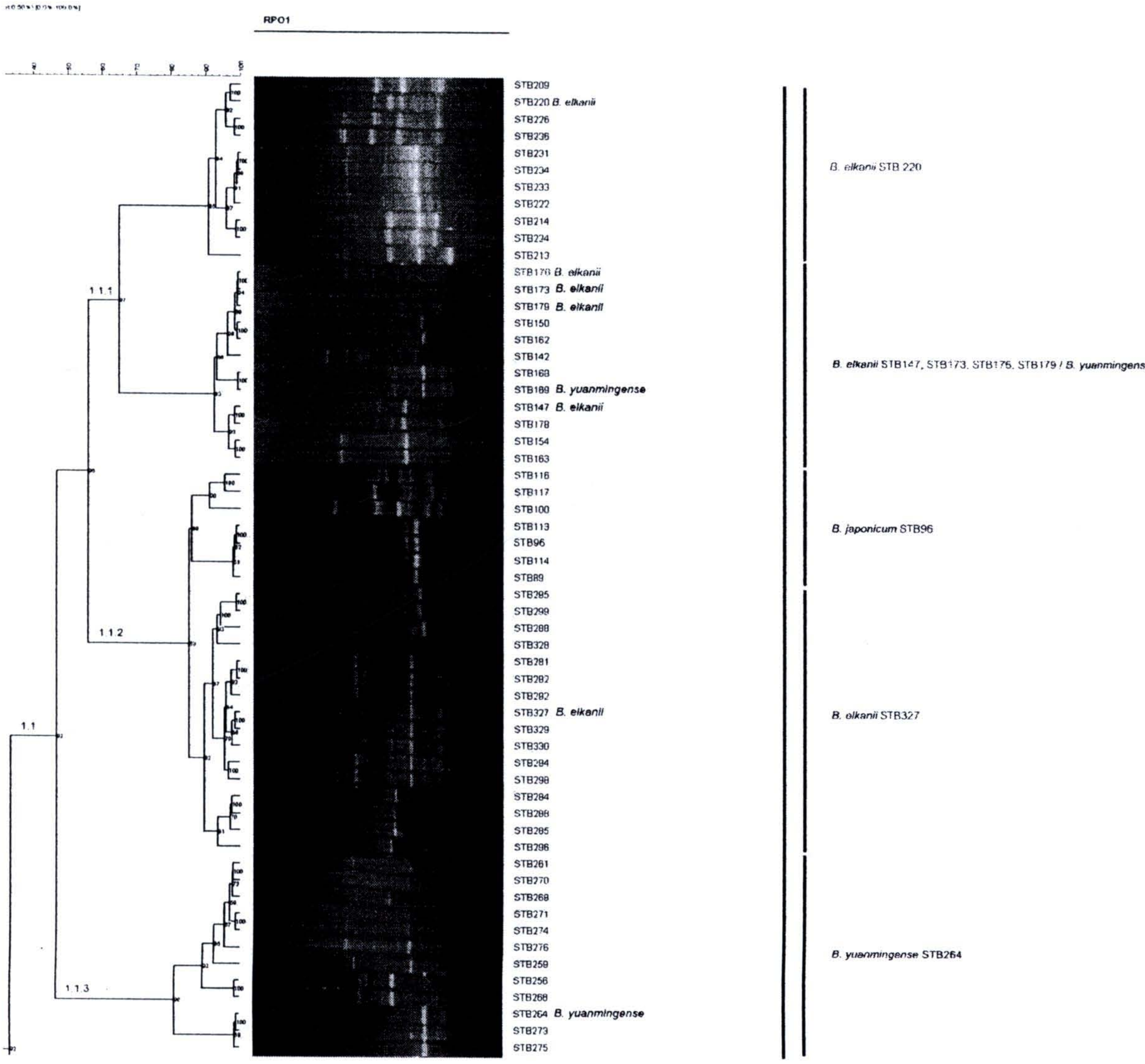


Figure 4.25 (b) Dendrogram constructed from DNA fingerprints using RPO1 as the primer. Detail is given in text.

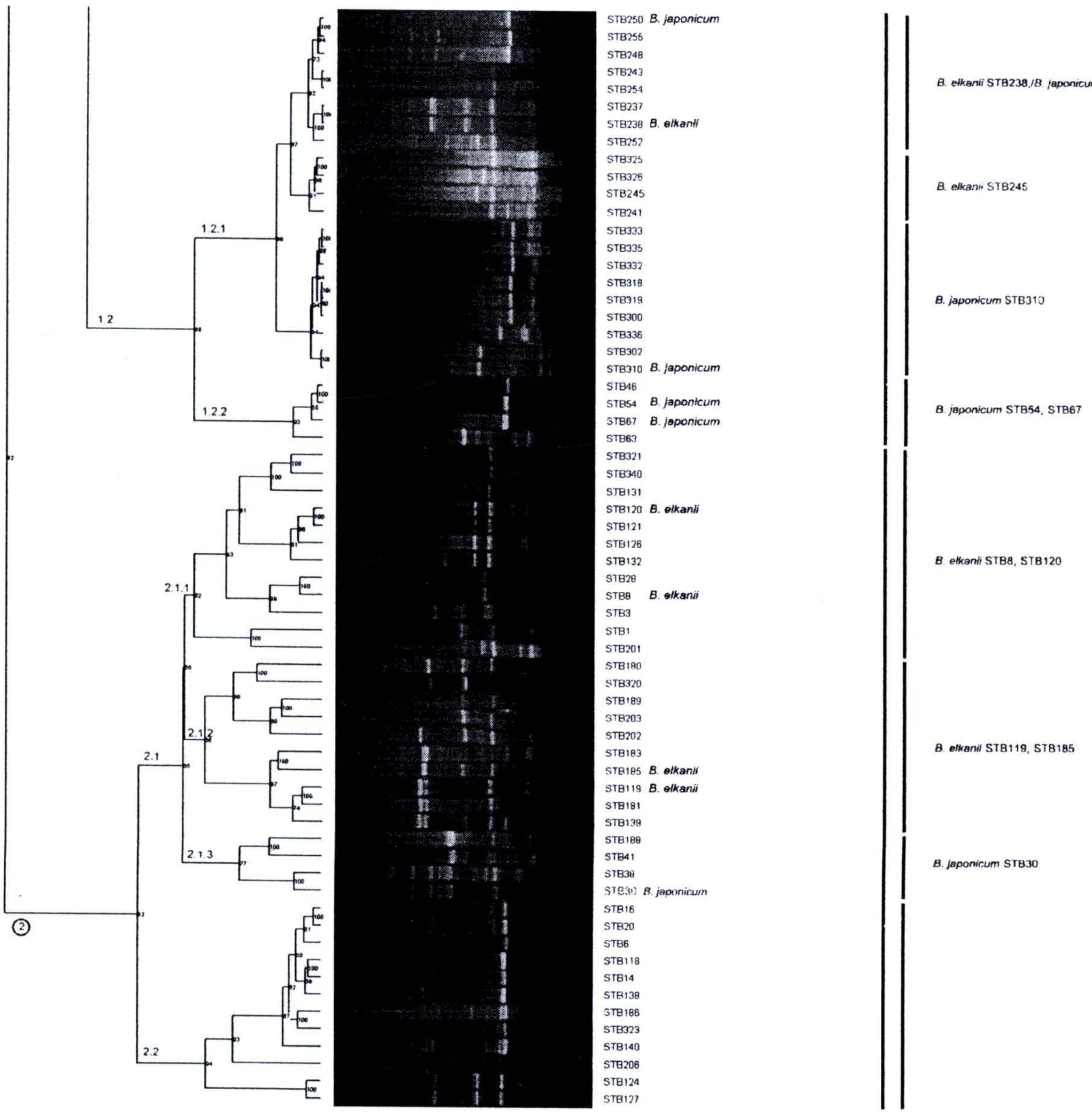


Figure 4.25 (b) Dendrogram constructed from DNA fingerprints using RPO1 as the primer. Detail is given in text.