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Production and Verification of Interspecific Hybrids in *Brassica* spp.

Chuanpis Aroonrungsikul[1], Kasem Piluek[2], Pornpan Phuprompun[1], Siriwan Burikam[1] and Siriporn Chumsangchotsakul[1]

ABSTRACT

Twenty days after cross pollinated Brassica spp. hybrid capsules were collected and embryo rescued into the modified MS. media supplement with 300 mg/l casein hydrolysate, 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l 6-benzylaminopurine (BA). The efficient production of interspecific hybrids between *B. campestris* x *B. juncea, B. Junceax B. campestris, B. campestris* x *B. oleracea* and *B. juncea* x *B. oleracea* were successfully regenerated to plantlets after 14 days *in vitro* culture. The hybrids were confirmed for fertility of pollen mother cell and the electrophoretic patterns of esterase (EST), malate dehydrogenase (MDH) and alcohol dehydrogenase (ADH) were observed between species by using polyacrylamide gel.

Keywords: interspecific hybridization, *Brassica* spp., embryo rescue, electrophoresis

INTRODUCTION

Recently, the progress of *in vitro* culture make it reasonable to consider interspecific hybridization as a valuable supplementary tool. It is already available at the various levels of breeding programme in vegetable crops. *Brassica* sp. is the one importance economic crop of Thailand and other tropical and subtropical countries. Seed production of chinese kale and cabbage are difficult in these region due to lack of low temperature. It is necessary to improve seed vernalized interspecific hybrids between chinese kale or cabbage and chinese cabage, transferring the seed vernalizing character of chinese cabbage into chinese kale or cabbage. In order to make the hybrids which may not require as low temperature for flowering induction. Therefore, the goals of interspecific hybridization are focused on the disease resistance breeding programme and the economic characters from one species to another (Namai *et al.*, 1980)

Embryo culture can be very helpful to overcome the barriers in carly stage of embryo development. The young embryos require different media depending on the stage of abortion or injury and their nutritive requirements are more complicated than mature embryo (Raghavan and Srivastava, 1982 and Rangan, 1982). Many studies tried to investigate the cytogenetical studies, chromosome and isozyme analyses of the hybrid of Cruciferae interspecific crosses (Inomata, 1980; Coulthart and Denfore, 1982 and Hossain *et al.*, 1988)

MATERIALS AND METHODS

Plant materials and vernalization

The materials used in the present experiment were similar to those as previously studied (Aroonrungsikul *et al.*, 1992) except 2 Japanese varieties, *B. campestris* (Hiroshimana) and *B. juncea* (Kobutakana) were used instead of *B. campestris* var. pekinensis (V_2) and *B. juncea* (Leaf Mustard Headtype). The plant materials were as follows: *B. oleracea* var. alboglabra (Leaf Kailaan), *B. campestris* var. pekinensis (Pak Choy), *B. campestris* var. parachinensis (Flowering Stalk Chinese Cabbage). All materials were introduced from Taiwan and Re-

1/ Central Laboratory and Greenhouse Complex, KURDI, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
2/ Dept. of Horticulture, Faculty of Agrilture, Kasetsart University, 10900 Thailand.

public of China.

The green plant of *B. oleracea* and seed of *B. campestris* and *B. juncea* were treated at 5 C temperature for 45 and 30 days, respectively and were synchronized for flowering periods. All treated plants were transferred into small pots containing sand, burn husk and organic manure mixture and were under 23-25 C with continuous illumination with 4000-6000 lux light intensity.

Embryo rescued culture

Twenty days after pollination, the hybrid capsules were collected and surface sterilized with 10 and 5% sodium hypochlorite. The embryos were rescued onto the modified MS medium then incubated under 25-28 C with alternated light period of 16/8 h/day at 3000 lux intensity. The modified MS medium was supplemented with 10% (V/V) coconut juice, 300 mg/l casein hydrolysate, 0.1 mg/l NAA and 0.1 mg/l BA and mixed with 30 g/l sucrose, 8 g/l agar adjusted at pH 5.8 (Hossain *et al.*, 1988). Length of capsule, number of embryo per capsule, number of proliferated hybrids were recorded. Only the proliferated hybrids were transplanted onto hormone free modified MS medium for two weeks. Afterwards, the fully developed hybrids were again transferred into the sterile mixture of sand : burn husk : manure at 1:2:1 ratio.

Slab gel electrophoresis

The thirth fully expanded leaf from apical meristem of hybrids and parents were taken for electrophoresis analyse. The analysis on polyacrylamide gels, 30-50 mg fresh weight of leaf tissue homogenized in 5 ml of cold extraction buffer in chilled mortar. Two extraction buffers were used to optimize resolution of isozyme bands : 0.1 M phosphate buffer (pH 7.5) and 0.1 M tris buffer (pH 8.2). Vertical slab gels electrophoresis were prepared from resolving gel contained 7.5% (W/V) polyacrylamide gel, 36.6% (W/V) Tris-hydroxymethyl (Tris), 0.23% (V/V) TEMED and 1 N HCl adjusted pH 8.9. Fifty microliters extracted samples were loaded in the wells then the contant current of 1.5 mA per well was applied for 3-4 h, temperature was controlled at 5-10 C. After electrophoresis, gels were stained for esterase (EST) alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH) (Sundberg and Glimelius, 1986).

Pollen fertility examination

First meiotic division of the hybrids and the parents were examined by staining pollen mother cells with acetocarmine. Pollen fertility percentage was estimied by average 20 pollen grains collected from blooming flowers of each plant and were stained with acetocarmine (Inmata, 1980).

RESULTS AND DISCUSSION

Crossing ability

The combination between female parent *B. campestris* var. *parachinensis* (V_4) with male parent *B. juncea* showed highest fertilization rate, but showed unproductive cross with male parent *B. oleracea* var. *alboglabra*. The crosses between female parents of *B. campestris* var. *parachinensis* (V_6) var. *chinensis*

Table 1 Development of interspecific hybrid capsules through in vitro culture of *Brassica* spp.

Combination[1]	Fertilization rate[2]	No.of hybrid capsule	No.of hybrid embryo	production rate[3]
$V_3 \times V_9$	30.14	54	238	4.41
$V_3 \times V_{12}$	26.90	53	239	4.51
$V_3 \times V_{13}$	35.00	49	317	6.47
$V_3 \times V_{10}$	17.56	23	86	3.74
$V_3 \times V_{11}$	32.43	12.	16	1.33
$V_4 \times V_9$	59.86	85	622	7.32
$V_4 \times V_{12}$	67.16	45	261	5.80
$V_4 \times V_{13}$	69.03	107	770	7.19

Table 1 Development of interspecific hybrid capsules through in vitro culture of *Brassica* spp. (con't)

Combination[1]	Fertilization rate[2]	No.of hybrid capsule	No.of hybrid embryo	production rate[3]
$V_6 \times V_9$	54.93	39	230	5.89
$V_6 \times V_{12}$	58.82	20	97	4.85
$V_6 \times V_{13}$	50.00	48	286	5.96
$V_6 \times V_{10}$	38.24	13	44	3.38
$V_6 \times V_{11}$	39.13	9	33	3.67
$V_7 \times V_9$	53.49	46	215	4.67
$V_7 \times V_{12}$	36.96	17	64	3.76
$V_7 \times V_{13}$	59.77	52	189	3.63
$V_7 \times V_{10}$	24.00	12	8	0.67
$V_7 \times V_{11}$	30.77	4	1	0.25
$V_9 \times V_3$	25.71	36	72	2.00
$V_9 \times V_4$	20.53	31	56	1.81
$V_9 \times V_6$	35.29	42	57	1.36
$V_9 \times V_7$	51.43	36	57	1.58
$V_9 \times V_{10}$	11.57	14	11	0.79
$V_9 \times V_{11}$	15.94	11	29	2.64
$V_{12} \times V_3$	17.65	7	9	1.29
$V_{12} \times V_7$	28.00	3	15	5.00
$V_{12} \times V_{10}$	17.65	3	8	2.67
$V_{13} \times V_3$	24.43	32	62	1.94
$V_{13} \times V_4$	20.33	25	41	1.28
$V_{13} \times V_6$	39.76	33	91	2.76
$V_{13} \times V_7$	61.64	45	136	3.62
$V_{13} \times V_{10}$	15.32	17	28	1.65

1 Combination :

V_3: *B. campestris* var. *pekinensis* (Pak Choy)
V_4: *B. campestris* var. *parachinensis* (Flowering Stalk Chinese Cabbage)
V_6: *B. campestris* var. *parachinensis* (Tsui-Sim Green Stalk)
V_7: *B campestris* var. *chinensis* (Large White Cabbage)
V_9: *B juncea* (Leaf Mustard)
V_{10}: *B oleracea* var. *alboglabra* (Taiwan : Leaf Kailaan)
V_{11}: *B oleracea* var. *alboglabra* (Rep. of China : Leaf Kailaan)
V_{12}: *B juncea* (Mustard Green)
V_{13}: *B juncea* (Kobutakana)

2 Fertilization rate $= \frac{\text{no. of capsule}}{\text{no. of pollinated flower}}$

3 Production rate $= \frac{\text{no. of hybrid embryo}}{\text{no. of hybrid capsule}}$

(V_7), var. *pekinensis* (V_3) and male parent *B. juncea*, *B. oleracea* obtained fertilization rate as shown in Table 1. Their reciprocal crosses revealed low fertilization rate except the male parent of *B. campestris* var. *chinensis* (V_9 x V_7, V_{12} x V_7 and V_{13} x V_7) which showed fertilization rate at 51.43, 28.00 and 61.64%, resp. (Table 1). The fertilization rate of the combinations of *B. campestris* x *B. oleracea* and *B. juncea* x *B. oleracea* varied were 17.56 - 39.13 and 11.57 - 17.65, but the production rate, examined at 20 days after pollination, were 0.25 - 3.74 and 0.79 - 2.67, resp. (Table 1). Previous work also found low production rate of hybrid between *B. campestris* x *B. oleracea*, while its reciprocal yielded higher production rate (*Aroonrungsikul* et al., 1992) The cross combination of *B. oleracea* var. *alboglabra* and *B. campestris* var. *pekinensis* obtained production rate only at 0.67% which was simila to the cross combination of *B. oleracea* var. *capitata* and *B. campestris* var. *pekinensis* (Hossain, *et al.*, 1988).

Embryo culture and plant regeneration

The modified medium of Murashige and Skoog supplemented with 10% (V/V) coconut juice, 300 mg/l casein hydrolysate, 0.1 mg/l NAA and 0.1 mg/l BA was effective for regenerating plantlets from hybrid embryos (Fig 1). The number of interspecific hybrid plants obtained in this investigation depended on the

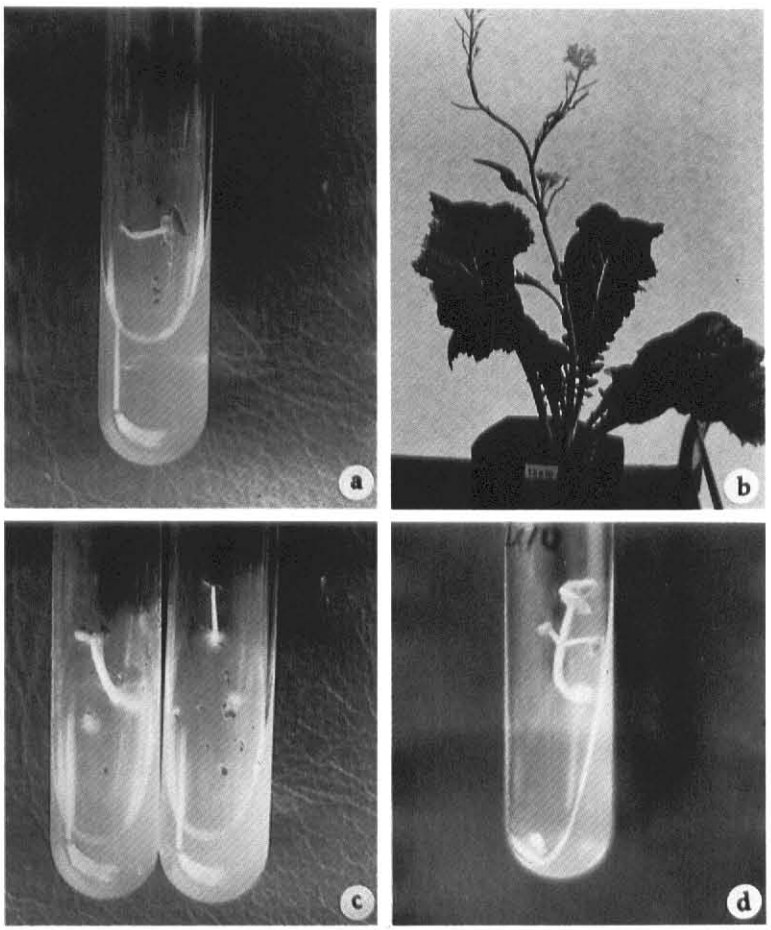

Figure 1 Interspecific hybrids obtained through embryo rescued of
(a) *B. juncea* x *B. oleracea* var. *alboglabra* 7 days after *in vitro* culture
(b) *B. juncea* x *B. oleracea* var. *alboglabra* 2 months after transferring in pot culture
(c) *B. juncea* x *B. campestris* var. *chinensis* (both tubes)
(d) *B. campestris* var. *parachinensis* x *B. oleracea* var. *alboglabra*

combinations. Among the interspecific hybridization, maxinum number of embryo per capsule was 7.68 ± 2.10 in *B. campestris* var. *parachinensis* (Flowering Stalk Chinese cabbage) x *B. juncea* (Leaf Mustard). While the hybrid embryo per capsule between female parent *B. campestris* var. *pekinensis* (Pak Choy) and *B. campestris* var. *parachinensis* (Tsui-Sim Green Stalk Cabbage) and male parent *B. juncea* and *B. oleracea* were 6.38 ± 2.07, 6.47 ± 1.92, 6.29 ± 1.82 and 5.38 ± 1.80 respectively (Table 2). But their reciprocal combinations showed a few number of embryo per capsule. This was also the case of the hybrid between *B. campestris* var. *chinensis* (Large White Cabbage) and *B. juncea* and *B. oleracea* as well. Aroonrungsikul *et al.* (1992b) reported that the hybrid *B. campestris* var. *chinensis* x *B. oleracea* var. *alboglabra* obtained 3.5 ± 1.60 embryos in a capsule and was successful in 57% hybrid plants. Similar combination could successfully regenerate 25-100% plantlets after developed from 1-8 number of embryo rescued and only 1-2 hybrid plants through further culture in modified MS medium with the supplements (Table 2). For reciprocal cross, no regenerated plant was observed. Hossain *et al*. (1988) investigated the cross between *B. oleracea* x *B. campestris* and found only 0.67 - 4.67 hybrid plant precentage. However no regenerated hybrid was observed for this combination in the work of Aroonrungsikul *et al* . (1992b).

The number of interspecific hybrid plants obtained, was under the influence of combination (Hossain *et al*., 1988). The maximum 44 hybrid plants were regenerated from hybrid between *B. juncea* (Kobutakana var.) x *B. campestris* var. *chinensis* (Large White Cabbage), reckoned as 26.99% hybrid plant obtained. The same female parent *B. juncea* crosed with male parent *B. campestris* var. *pekinensis* (Pak-Choy) could produce only 5 plants or 8.06% obtained. While, the Leaf mustard (another *B. juncea*) crossed with Large White Cabbage and Pak-Choy

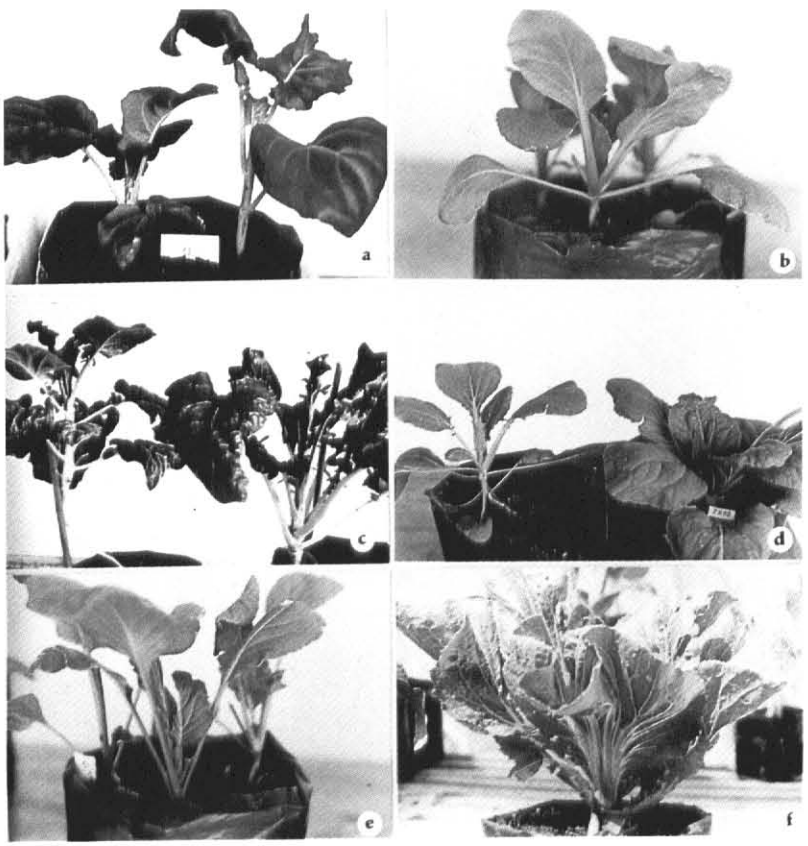

Figure 2 Interspecific hybrids and parents of *Brassica* spp. (a,c and e) *B. oleracea*, hybrid and *B. campestris* var. *parachinensis*; (b,d and f) *B. campestris* var. chinensis, hybrid and *B. juncea*.

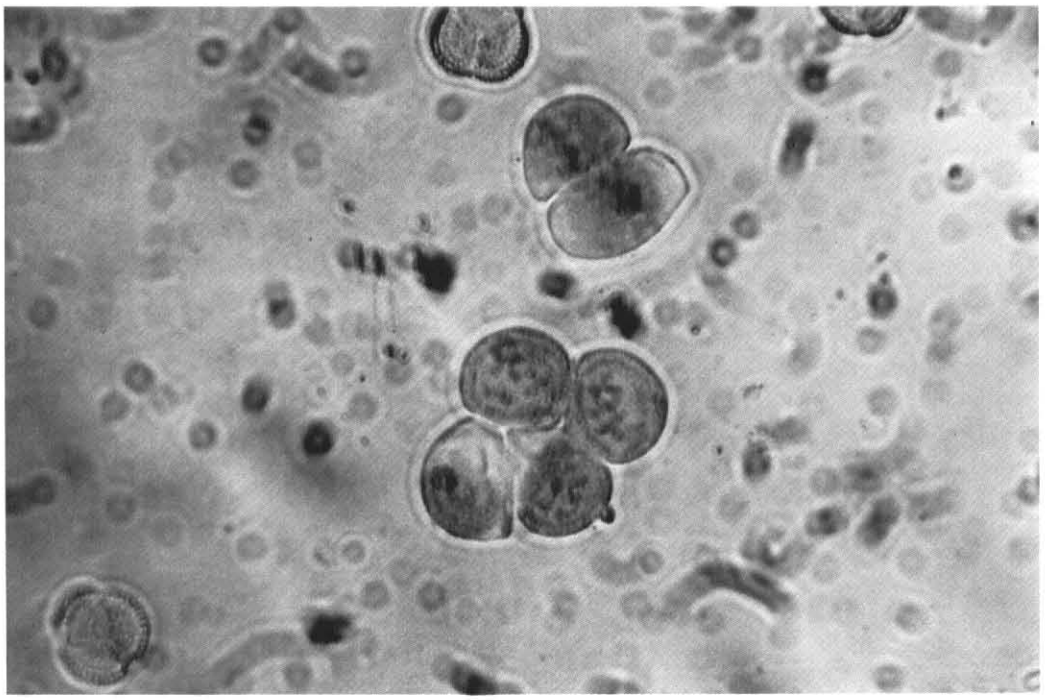

Figure 3 Pollen mother cell development in hybrid between *B. campestris* (Hiroshimana) x *B. juncea* (Kobutakana) showed unequal division of tetrads.

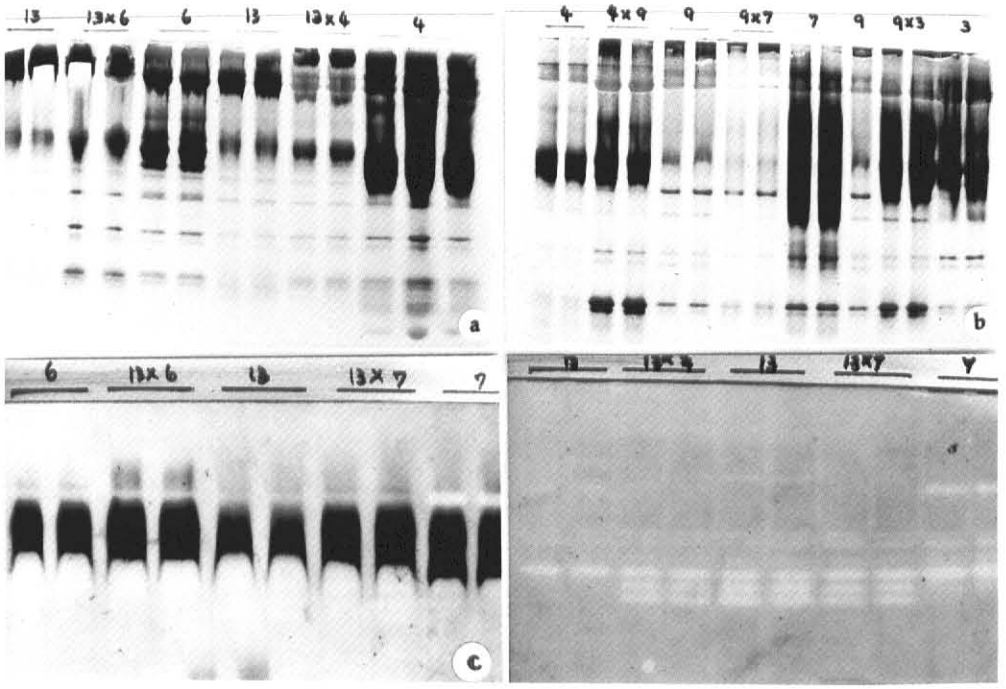

Figure 4 Isozyme analysis of the interspecific hybrids and parents : (a and b) esterase isozyme patterns. (c) malate dehydrogenase isozyme pattern and (d) alcohol dehydrogenase isozyme pattern.

Table 2 Interspecific hybrids through embryo culture of *Brassica* spp.

Combination[1]	Lingth of capsule (mm.)	No.embryo per capsule	No.embryo rescued	No.hybrid plantlet	%hybrid[2] plant obtained
V3 x V12	16.67±2.31	6.38±2.07	239	1	0.42
V3 x V13	19.14±2.39	6.47±1.92	317	6	1.89
V3 x V10	13.74±1.95	5.38±1.80	86	1	1.16
V4 x V9	32.00±2.93	7.68±2.10	622	13	2.09
V3 x V13	29.68±2.75	5.42±1.89	770	1	0.13
V6 x V10	19.75±0.87	6.29±1.82	44	3	6.82
V7 x V9	22.87±2.38	2.00	215	7	3.26
V7 x V13	21.31±2.25	3.84±1.69	189	5	2.65
V7 x V10	15.50±1.98	2.00±1.00	8	2	25.00
V7 x V_{11}	20.50±1.79	1.00	1	1	100.00
V5 x V13	22.77±2.05	3.7±1.09	37	16	43.24
V9 x V3	24.53±2.30	3.43±1.67	72	10	13.89
V9 x V4	20.19±2.57	3.73±1.84	56	1	1.79
V9 x V6	19.02±2.07	2.85±1.38	57	3	5.26
V9 x V7	20.25±2.22	2.76±1.25	57	19	33.33
V13 x V3	18.28±1.63	2.70±1.42	62	5	8.06
V13 x V4	17.52±1.83	2.16±1.19	41	9	21.95
V13 x V6	19.00±1.82	3.21±1.68	91	20	21.98
V13 x V7	20.27±2.04	4.18±1.63	163	44	26.99
V13 x V10	13.59±1.97	3.38±1.31	28	5	17.86

1 Combination :

V3: *B. campestris* var. *pekinensis* (Pak Choy)
V4: *B. campestris* var. *parachinensis* (Flowering Stalk Chinese Cabbage)
V5: *B. campestris* var. *pekinensis* (Hiroshimana)
V6: *B. campestris* var. *parachinensis* (Tsui-Sim Green Stalk)
V7: *B campestris* var. *chinensis* (Large White Cabbage)
V9: *B juncea* (Leaf Mustard)
V10: *B oleracea* var. *alboglabra* (Taiwan : Leaf Kailaan)
V11: *B oleracea* var. *alboglabra* (Rep. of China : Leaf Kailaan)
V12: *B juncea* (Mustard Green)
V13: *B juncea* (Kobutakana)

2 % hybrid plant $= \frac{\text{No. of hybrid plant}}{\text{No. of embryo explant}} \times 100$

Table 3 Leaf charactors, plant height and petiole length of some interspecific hybrids.

Combination[1]	leaf					Petiole length[2]	Plant height[2]
	Length[2]	Width[2]	Incision[3]	Margin[4]	Colour		
V4 x V9	14.49±1.52	8.41±0.96	S	E	Green	7.11±2.06	21.62±5.36
V6 x V10	10.80±2.65	9.17±0.76	L	E	Green	9.83±2.02	22.75±7.42
V7 x V9	12.93±1.90	7.95±2.01	L	E	Light green	5.08±0.49	23.75±1.06
V7 x V11	11.38±1.03	10.00±1.08	L	E	Green	6.68±0.72	27.00
V9 x V3	21.00±2.35	12.00±1.22	L	C	Light green	6.00±3.08	17.00
V9 x V7	12.67±1.04	8.50±0.50	L	C	Light green	4.67±1.04	23.10±7.41
V13 x V4	14.31±2.71	7.88±1.51	L	C	Green	5.59±0.99	22.20±2.68
V13 x V6	18.70±2.61	8.96±1.43	L	D	Green	5.86±0.96	25.94±2.03
V13 x V7	15.94±3.53	9.60±1.87	L	C	Green	5.33±1.59	20.77±8319
V13 x V10	11.88±1.55	6.88±0.85	L	E	Green	7.00±1.00	18.00

1 combination :

V3 = *B. campestris* var. *pekinensis*
V4 = *B. campestris* var. *parachinensis*
V6 = *B. campestris* var. *parachinensis*
V7 = *B. campestris* var. *chinensis*
V9 = *B. juncea*
V10 = *B oleracea* var. *alboglabra*
V11 = *B oleracea* var. *alboglabra*
V13 = *B. juncea*

2 length in cm
3 S = sinuate, L = lyrate
4 E = entrie, C = crenate, D = dentate

Sources : IBPGR (1990)

Table 4 Pollen fertility percentage of some interspecific combinations

Combination	Pollen fertility
V4 x V12	51.58
V7 x V12	35.50
V6 x V_9	16.30
V3 x V13	49.00
V13 x V10	56.00

V3 : *B. campestris* var. *pekinensis* (Pak Choy)
V4 : *B. campestris* var. *parachinensis* (Flowering Stalk Chinese Cabbage)
V6 : *B. campestris* var. *parachinensis* (Tsui-Sim Green Stalk)
V7 : *B. campestris* var. *chinensis* (Large White Cabbage)
V9 : *B. juncea* (Leaf Mustard)
V10 : *B. oleracea* var. *alboglabra* (Taiwan : Leaf Kailaan)
V12 : *B. juncea* (Mustard Green)
V13 : *B. juncea* (Kobutakana)

could yield 19 and 10 plants or 33.33 and 13.89%, repectively (Table 2). The observed phenomenon was due to the different races, cultivars or species, the pollen fertility, the meiotic pairing and hybrid stabilization (Snogerup, 1980). Reciprocal combination of female parent *B. oleracea* crossed with both *B. campestris* and *B. juncea* were not successfully regenerated in this experiment. Female parent, *B. campestris* was more probable to cross with male parent *B. juncea* than *B. oleracea*. In the recent studies of Inomata (1978 a and 1978 b), the hybrids between *B. campestris* x *B. oleracea* could be produced comparatively by *in vitro* culturing of excised ovaries. The lower rate of success in producing crosses of *B. oleracea* as the male parent and unproductivity of reciprocal in this study may be due to a strong incompatibility of *B. oleracea* which caused the pollen tube to fail to penetrate into the micropyle.

Characteristics of hybrid plants

Most of the hybrid plant traits were intermediate between the female and male parents (Fig 2). The hybrid plant between *B. campestris* var. *chinensis* x *B.*

juncea and Hiroshimana x Kobutakana yield spreading type, whereas *B. campestris* var. *parachinensis*, var. *pekinensis* x *B. juncea*, *B. oleracea* were erect type. The main distinguishing traits of hybrid were leaf : shape, incision, size and colour. All these characters varied greatly in comparison with their parents (Table 3). The hybrids of *B. campestris* x *B. campestris* x *B. oleracea*, *B. juncea* x *B. campestris* and *B. juncea* x *B. oleracea* produced yellow flowers, whereas hybrid of *B. oleracea* x *B. campestris* had white flowers. Hossain, *et. al.*, (1988) reported *B. oleracea* var. *capitata* x *B. campestris* var. *pekinensis* had yellow flowers and white flowers from hybrid *B. oleracea* var. *alboglabra* x *B. campestris* var. *pekinensis* and intergeneric hybrid of *B. oleracea* var. *capitata* x *Raphanus sativus*.

Pollen fertility of hybrids

The hybrid pollen fertility was observed from pollen mother cell using acetocarmine stain. Both crosses of *B. campistris* var. *parachinensis* x *B. juncea* showed 16.30 and 51.58% fertility. The hybrids between female parents of *B. campestris* var. *chinensis* and var. pekinensis crossed with male parents *B. juncea* (Kobutakana and Mustard Green) developed the anthers and showed 35.5 and 49% pollen fertility (Table 4) with dyads and unequal division of tetrads (Fig 3). No plant regenerated from crosses *B. oleracea* (female parent) with *B. campestris* or *B. juncea* (male parent) but hybrid between *B. juncea* and *B. oleracea* obtained mean pollen fertility at 56% and over 90% seed set after colchicine treatment. Inomata (1980) found *B. campestris* x *B. oleracea* pollen normally development in most hybrids and no fertile pollen grain was observed in the degenerated anther. The hybrid plants had normal pollen grains and range of pollen fertity varied from 0 to 27%

Isozyme analysis

Electrophoretic genetype of various isozyme pattern in hybrids between *B. campestris* x *B. juncea* and its reciprocal, *B. campestris* x *B. oleracea* and *B. juncea* x *B. oleracea* were observed. Esterase (EST), (E.C.3.1.1.2); malate dehydrogenase (MDH), (E.C.1.1.1.37) and alcohol dehydrogenase (ADH), (E.C.1.1.1.1) banding patternes of parents and hybrids were distincted (Fig 4). The hybrids bands were different to bands from parental plants, such as EST and ADH pattern of *B. juncea* (Kobutakana) x *B. campestris* var. *parachinensis* (both), ADH and MDH patterns of *B. juncea* (Kobutakana) x *B. campestris* var. *chinensis*. EST, ADH and MDH pattern of *B. juncea* (Leaf Mustard) *B. campestris* var. *pekinensis* and var. *chinensis*. EST and MDH patterms of reciprocal cross between *B. campestris* var. *parachinensis* x *B. juncea* (Leaf Mustard) showed similar characferistics as well. Having investigated electrophoretic pattern of seed proteins and seed enzyme, of certain species and cultivars of vegetable Nakamura (1977) reported that it was necessary to use at least two or three kinds of enzymatic patterns for full distinguish by EST, ADH, peroxidase, glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH) and carbonic anhydrase (CA). The report of Aroonrungsikul *et al.* (1992 a) showed the electrophoretic patterns of EST, MDH, glutamic oxaloacetic transaminase (GOT) and glucose-6-phosphate dehydrogenase (G-6P) could be valuble for *Brassica* species and cultivars detection.

CONCLUSION

Vernalization of *Brassica* spp. successfully hastened flowering and crosses pollination could carry on under growth cabinet. *In vitro* embryo rescued culture at 20 days after pollination was considered to be useful for the development of interspecific hybrid between *B. campestris* x *B. juncea*, *B. juncea* x *B. campestris*, *B. campestris* x *B. oleracea* and *B. juncea* x *B. oleracea*. The modified MS media supplemented with 10% (V/V) coconut juice, 300 mg/l casein hydrolysate, 0.1 mg/l NAA, and 0.1 mg/l BA was effective for regenerating the hybrid embryos. Low pollen fertility percentage of PMC of interspecific hybrids were obtained and the trits of hybrids were incorpotated from their parental plants. The slab gel electrophoresis of EST, ADH and MDH pattern could be used for the hybrids varietal confirmation.

LETERATURE CITED

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