Micropropagation of Mustard (*Brassica* spp.) from Leaf Explants

H. Basak, B.K. Biswas, M.A.K. Azad, M. Arifuzzaman* and F. Sharmeen

Department of Genetics and Plant Breeding, Faculty of Agriculture
Hajee Mohammad Danesh Science and Technology University
Dinajpur 5200, Bangladesh

**Corresponding author. Email: arif_gpb@yahoo.com*

Abstract

The experiment was carried out in the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur during November, 2008 to May, 2009 with a view to study *in vitro* regeneration of *Brassica* spp. from leaf explant. Leaf segments of the four genotypes of *Brassica* viz. BARI Sarisha-9, BARI Sarisha-11, Tori-7 and Sampad were cultured on MS medium with different concentrations and combinations of hormones. The range of percent callus induction was 55-100%. Among the four varieties BARI Sarisha-9 showed early callusing (6.22 days) with maximum rate of callus induction (96.11%). Early and maximum rate of callusing appeared in MS+1.5 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg $L^{-1}AgNO_3$ for all genotypes. Shoot regeneration ranged from 50% to 78.33% and BARI Sarisha-9 had the highest percentage of shoot regeneration (72.22%). Early and maximum rate of regeneration was found in MS+3.5 mg L^{-1} BAP+0.5 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ for all the genotypes. The highest number of roots per shoot was counted in BARI Sarisha-9 (76.67%) on $^1/_2$MS with 0.5 mg L^{-1} NAA. Considering the overall performance, genotype BARI Sarisha-9 appeared the best for callus formation, shoot regeneration and root formation.

Keywords: mustard, *in vitro* regeneration, leaf, hormone

Introduction

Brassica is the third most important edible oil sources in the world after soybean and palm (Piazza and Foglia, 2001; Walker and Boot, 2001). About 13.2% of the World's edible oil supply comes from this crop (Downey and Robbelen, 1989). In Bangladesh it remains top in the list in respect of area and production, among the oil crops grown in the country (Mondol and Wahhab, 2001). It covers 61.2% of the total oilseeds acreage of the country and 52.6% of the total production (BBS, 1999). The average yield of *Brassica* oilseed in Bangladesh is around 740 kg ha^{-1} (Chowdhury and Zulfikar, 2001; Mondal and Wahhab, 2001).In the recent years, there has been declining trends in both acreage and productions of oil crops in Bangladesh (BBS, 2004). Bangladesh is facing acute shortage in edible oils (BBS, 2003). At present the oilseed production is about 0.254 million ton, which covers only 40% of the domestic need (FAO, 2001). As a result, more than 50% requirement of oil has been imported every year by spending huge amount of foreign currency involving over Tk. 2820 crore (BBS, 2003)

The conventional breeding methods are most widely used for crop improvement. But in practical situations, these methods have to be supplemented with plant tissue culture techniques, either to increase their efficiency or to achieve the objectives, which in not possible through the conventional methods. To improve the characters of agronomic importance in *Brassica*, the conventional breeding methods were tried but they were not very successful due to high degree of segregation through cross-pollination and

unavailability of suitable wild germplasm of *Brassica*. Moreover, conventional breeding program is time consuming, laborious and needs wise selection of desirable traits.

The regeneration of plants from tissue culture is an important and essential component of biotechnological research, and sometimes is required for the genetic manipulation of plants. Tissue culture techniques may also be utilized conveniently to overcome incompatibility barrier through fusion of protoplasts from vegetative cells of interspecific, intergenceric and interfamiliar group (Rao and Chadha, 1986; Rao, 1985). During the last decades, considerable efforts have been made through out the world to develop *in vitro* technique for regeneration of *Brassica*. The regeneration of plants has been successful in *Brassica* spp. using different explant, such as petiole, cotyledon, stem and shoot tip. Shoot regeneration, rooting and survival of plants were high in plants regenerated from cotyledon and hypocotyl explants of Indian mustard cultivars (Bhalla *et al*., 2001). Shoot tip explant of Brassica were reported to be effective for initiating shoots and roots (Zhang *et al*., 1989).

Leaf explant culture is an alternative technique for initiating shoots and roots of *Brassica*, and also less time consuming. Moreover, a small part of leaf is enough for the regeneration of *Brassica*. *In vitro* response of young leaf is also remarkable and produces shoot and roots rapidly. There are some reports on *in vitro* leaf culture of cabbage but there was limited report on in vitro leaf culture of *Brassica*. Therefore, the objectives of the present study were to investigate the callus induction and regeneration potentiality of selected varieties of *Brassica* in different hormone combinations and to identify a suitable protocol for *in vitro* regeneration of plantlets of *Brassica* spp. through leaf explant cultures.

Materials and Methods

Location, Time Duration and Year

The study was conducted at Tissue culture laboratory of the Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh. during the period from November, 2008 to May, 2009.

Experimental Materials Four different genotypes of *Brassica* spp. such as BARI Sarisha-9, BARI Sarisha-11, Tori-7 and Sampad were used in the present investigation to study different parameters associated with plant generation. The seed materials of *Brassica* genotypes were collected from the Bangladesh Agricultural Development Corporation (BADC), Dinajpur, Bangladesh.

Sterilization of Experimental Materials

Matured seeds were washed in running tap water for 3-5 minutes with two to three times to remove the level of surface organism. The floating seeds were discarded. Later the seeds were dipped in 70% ethyl alcohol for 3-5 minutes with gentle shaking followed by washing with sterile distilled water. Surface disinfections was done by the use of sodium hypochlorite solution (1% active chlorine) containing 1-2 drops of tween-20 for ten minutes with gentle shaking and then rinsed five times in sterile distilled water.

Cultural Techniques

Sterilized seeds were placed onto seed germinating MS (Murashige and Shoog, 1962) medium in culture vials. In each vials 8-12 seeds were incubated. From 6-7 days old seedling leaves were cut into 12-15 pieces with 2-5 mm in length by using sterilized surgical blades. Then in each vials four leaf segments were placed gently, which contained sterilized MS medium with various combinations and concentrations of growth regulators like 2, 4-D(2,4-dichlorophenoxyacetic acid), NAA(1-Naphthaleneacetic acid) and $AgNO_3$.

After 20-25 days of inoculation of explants, the calli attained desirable size for transfer to regeneration medium. Then, they were removed aseptically from the vials to new vials containing sterilized induction medium using forceps. The transfer was done inside the laminar airflow cabinet. The sub-cultured media used in the present investigation were MS (Murashige and Skoog, 1962) medium containing different combinations and concentrations of BAP (6-Benzylaminopurine), NAA and $AgNO_3$. Repeated subcultures were also done at an interval of 15-20 days and incubated

under the same temperature as mentioned previously for maintenance of calli and organogenesis. The sub-cultured calli continued to proliferate and differentiated into shoots. When these shoot grew about 2-3 cm in length were separated from each other and again cultured individually on vials containing sterilized root induction medium to induce root. The vials containing plantlets were incubated as mentioned previously.

Recording of Data

To investigate the effect of different treatments and response of different varieties to callus induction and plant regeneration, data were recorded on different parameters like days to callus, shoot and root initiations, number of explants showing callus, shoot and shoot with root regeneration. Total established plants were calculated based on the number of plantlets place in the pots and the number of plants finally established or survived:

$$Percent\ \ plant\ establishment = \frac{No.of\ establishment\ \ plantlet}{Total\ no.of\ \ plantlets} \times 100$$

Statistical Analysis

The program MSTATC was used to analyze the data for different parameters recorded in the present study wherever applicable. The experiment was conducted in incubation room of tissue culture laboratory and arranged in Completely Randomized Design (CRD). The analysis of variances for different parameters was performed band means were compared by the Least Significant Difference (LSD) Test at 5% level of significance.

Results and Discussion

Callus Induction

Mean values of genotypes and treatments on callus inducing characters like total number of callus induction, percent (%) callus induction and days to callus initiation were found statistically significant. The results of the different genotypes observed in the present study are presented in Table 1. Leaf explants started callus initiation by changing their shape after six days of incubation, and callus formation was completed within nine days of incubation. The highest percentage (96.11%) of callus induction was found in the variety of BARI Sarisha-9 followed by Tori-7 (88.89%) (Figure 2), BARI Sarisha-11 (76.11%) and Sampad (62.78%) (Table 1). BARI Sarisha-9 showed callus initiation early (6.22 days) in comparison to other genotypes such as Tori-7 (6.667 days), BARI Sarisha-11 (7.67 days) and Sampad (8.00 days). But, there is no significant difference from BARI Sarisha-9 to Tori-7 in case of callus initiation. Callus initiation was late in Sampad (8.00 days) and there was also no significant difference between BARI Sarisha-11 and Sampad.

Total number of callus induction was highest (17.33) with high percentage (86.67%) of callus induction in T_2 (MS+1.5 mg L^{-1} 2,4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$) treatment (Figure 2) followed by T_3 (MS+2.0 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$) and T_1(MS+1.5 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$) (Table 1).T_3 (MS +2.0 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$) treatment required maximum (7.42 days) for callus initiations followed by T_1 (7.147 days) and T_2 (6.83 days).

Effects of phytohormone×variety interactions on different parameter such as number of explants with callus, percent callus induction and days required for callus initiations were found as highly significant (data not shown). The maximum number of explants showing callus was found in BARI Sarisha-9 genotype with (T_3)MS+2.0 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ and (T_2) MS+1.5 mg L^{-1} 2,4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ (Figure 1). Early callusing was observed between treatments (T_3) MS+2.mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ and the genotype BARI Sarisha-9 (5.67 days) followed by the genotype Tori-7×T_1 (6.00 days) and BARI Sarisha-9×T_2 (6.00 days). All the genotypes gave satisfactory results under (T_2) MS+1.5 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA +2.0 mg L^{-1} $AgNO_3$ treatments except Tori-7. From the above results, it may be concluded that BARI sarisha-9 with T_2 (MS+1.5 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$) showed the best performance on callus induction. This result was in agreement with those obtained by Chen et al. (2005), Shanti et al. (2002) and Dhawanet et al. (2004).

Table 1 Effect of varieties and treatments on callus induction of *Brassica* spp.

Variety	Total no of explants inoculated	Total no. of callus induction	% callus induction	Days required for callus induction
BARI Sarisha-9	20	19.22 a	96.11 a	6.22 c
BARI Sarisha-11	20	15.22 c	76.11 c	7.67 ab
Tori-7	20	17.56 b	88.89 b	6.67 bc
Sampad	20	12.56 d	62.78 d	8.00 a
Treatment				
T_1	20	14.92 c	75.42 b	7.17 a
T_2	20	17.33 a	86.67 a	6.83 a
T_3	20	16.17 b	80.83 ab	7.42 a

Means in a column followed by uncommon letter(s) varied significantly at 5 % level of LSD.
T_1=MS+1.0 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$, T_2= MS+1.5 mg L^{-1}2, 4-d+0.3 mg L^{-1} NAA+2.0 mg $L^{-1}AgNO_3$, T_3= MS+2.0 mg L^{-1}2, 4-d+0.3 mg L^{-1} NAA+2.0 mg $L^{-1}AgNO_3$

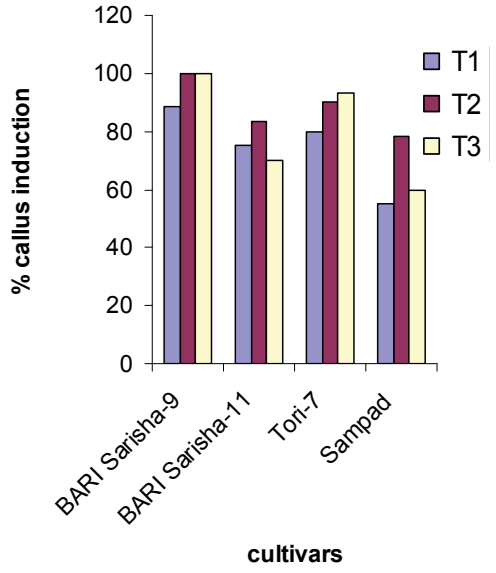


Figure 1 Combined effect of different treatments on percent callus induction from leaf explants of four *Brassica* spp.

Organogenesis via Callus

Leaf segments of four *Brassica* species were cultured on MS medium supplemented with different concentrations of BAP (3.0, 3.5 and 4.0 mg L^{-1}) with constant concentration of NAA (0.5 mg L^{-1}) and $AgNO_3$ (2 mg L^{-1}) in order to induce shoot from unorganized calli. The various morphogenic response of calli to different concentration of BAP in the medium has been observed and results are presented Table 2.

Among the four genotypes, BARI Sarisha-9 showed best performance (78.33%) on percent shoot regeneration in MS+3.5 mg L^{-1}BAP +0.5 mg L^{-1} NAA+2.0 mgL^{-1} $AgNO_3$ (T_2) (Table 2). In contrast, BARI Sarisha-11 and Sampad showed lowest performance (50%) on percent shoot regeneration with MS+4.0mg L^{-1} BAP+0.5 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ (T_3). Days required for shoot initiation was minimum (43.67 days) on the interactions of MS+4.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ (T_3) with BARI Sarisha-9 (Figure 2) and maximum (52 days) on the interactions MS+3.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ (T_1) with Sampad (Table 2). All the genotypes showed satisfactory results against MS+3.5 mg L^{-1} BAP+0.5 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ (T_2) treatment. From the above results, it may be concluded that T_2 (MS+3.5 mg L^{-1} BAP+0.5 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$) showed the best performance on shoot regeneration. The present findings showed conformity with those of Shanti et al. (2002), Patil et al. (2006), Zhang et al. (2002), Shinget al. (2007).

Root Induction

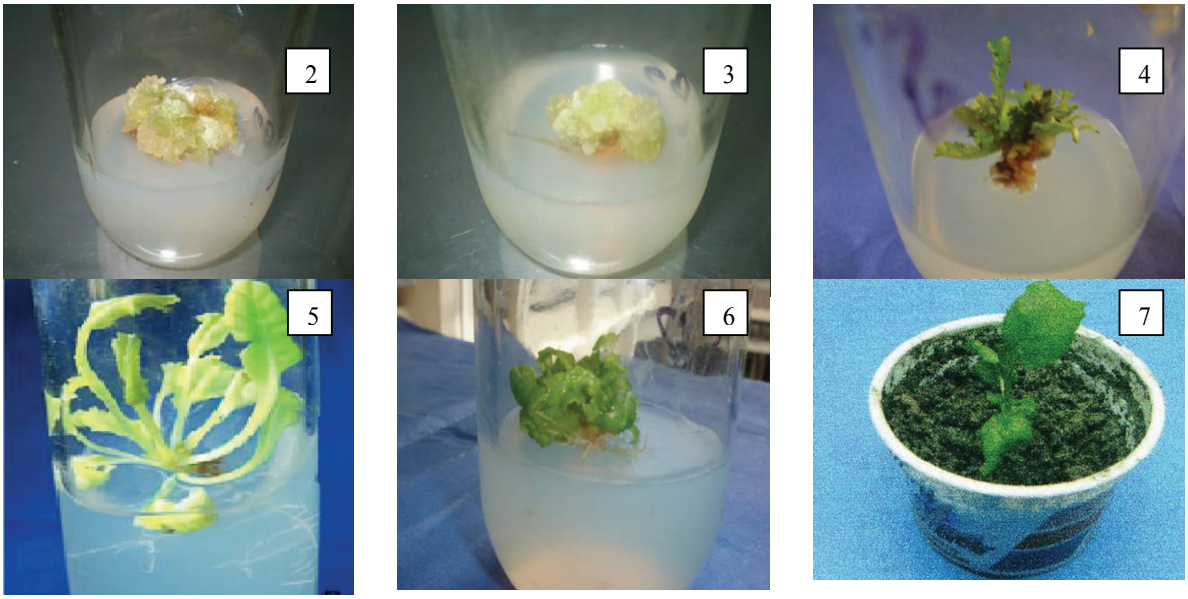
Results related for the characters of root regeneration such as percent root formation and days required to root formation in different concentrations of MS and NAA showed significant variations. The results are presented in Table 3.

Among the four genotypes, BARI Sarisha-9 showed best performance (80.00%) on percent root regeneration in ½MS (T_1) and ½MS+0.5 mg L^{-1} NAA (T_2) (Figure 2). But in contrast, Sampad showed the lowest performance (23.33%) on percent root regeneration with ½MS+1 mg L^{-1} NAA (T_3) (Table 3). Figure 2 shows moderate (53.33%) rooting on Tori-7 genotype under ½MS+

Table 2 Combined effect of variety and hormone concentrations on shoot formation of *Brassica* spp.

	Hormone ×variety	Total no. of calli inoculated	Total no. of calli showing shoot	% shoot formation	Days required for shoot initiation
T_1	BARI Sarisha-9	20	13.00 c	65.00 c	45.00 e
	BARI Sarisha-11	20	10.00 f	50.00 f	46.33 d
	Tori-7	20	14.33 b	71.67 b	46.33 d
	Sampad	20	11.33 e	56.67 e	52.00 a
T_2	BARI Sarisha-9	20	15.67 a	78.33 a	43.67 f
	BARI Sarisha-11	20	12.00 d	60.00 d	45.33 e
	Tori-7	20	13.00 c	65.00 c	45.00 e
	Sampad	20	12.00 d	60.00 d	49.67 b
T_3	BARI Sarisha-9	20	14.67 b	73.33 b	42.67 g
	BARI Sarisha-11	20	10.00 f	50.00 f	47.67 c
	Tori-7	20	12.33 d	61.67 d	45.67 de
	Sampad	20	10.00 f	50.00 f	48.00 c

Means in a column followed by uncommon letter(s) varied significantly at 5 % level of LSD.
T_1=MS+1.0 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$, T_2= MS+1.5 mg L^{-1}2, 4-d+0.3 mg L^{-1} NAA+2.0 mg $L^{-1}AgNO_3$, T_3= MS+2.0 mg L^{-1}2, 4-d+0.3 mg L^{-1} NAA+2.0 mg $L^{-1}AgNO_3$



Figures 2 Development of callus from the genotype of BARI Sariaha-9 using MS+1.5 mg L^{-1} 2, 4-d+0.3 mgL^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ (2), development of callus from the genotype of Tori-7 using MS+1.5 mg L^{-1} 2, 4-d+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ (3), initiation of shoot from the callus of BARI Sarisha-9 genotype using MS+4.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ (4), root initiation from regenerated shoot of BARI Sarisha-9 in ½ MS +0.5 mgL^{-1} NAA (5), root initiation from regenerated shoot of Tori-7 in ½ MS +0.5 mgL^{-1} NAA (6), and survived plant after hardening of BARI Sarisha-9 derived from leaf explants (7).

0.5 mg L^{-1} NAA (T_2). Days required for root initiation was minimum (11.33 days) on the interaction of ½ MS +0.5 mg L^{-1} NAA (T_2) with BARI Sarisha-9 and maximum (19.00 days) on the interaction ½MS (T_1) and ½MS+1 mg L^{-1} NAA (T_3) with Sampad (Table 3). Similar results also reported by Chen et al. (2005), Cheng et al. (2001) and Kharb and Chowdhury (1995).

Table 3 Effect of hormone×genotype interaction on root formation of *Brassica* spp. using 10 shoots

Hormone×genotype		No. of shoots with root	Root formation (%)	Days required root formation
T_1	BARI Sarisha-9	8.0a	80.0a	14.0g
	BARI Sarisha-11	5.3de	53.3de	15.7e
	Tori-7	5.7cd	56.7cd	14.7f
	Sampad	3.0f	30.0f	19.0a
T_2	BARI Sarisha-9	8.0a	80.0a	11.3h
	BARI Sarisha-11	6.0c	60.0c	16.7d
	Tori-7	5.3de	53.3de	15.0f
	Sampad	3.0f	30.0f	17.7c
T_3	BARI Sarisha-9	7.0b	70.0b	13.7g
	BARI Sarisha-11	5.0e	50.0e	18.3b
	Tori-7	6.0c	60.0c	13.7g
	Sampad	2.3g	23.3g	19.0a

Means in a column followed by uncommon letter(s) varied significantly at 5 % level of LSD.
T_1= Half strength MS medium, T_2= Half strength MS medium containing 0.5 mg L^{-1} NAA,
T_3=Half strength MS medium containing 1 mg L^{-1} NAA

Table 4 Comparative survivability rate of regenerates obtain from leaf discs of the four genotypes of *Brassica* spp.

Planting condition	Genotypes name	Number of plants transplanted	Number of plants survived	Survival rate (%)
In pot	BARI Sarisha-9	10	6	60
	BARI Sarisha-11	10	4	40
	Tori-7	8	5	62.5
	Sampad	8	3	35.5

Establishment of Plantlet

After sufficient development of root system, the small plantlets were taken out from culture vessels without any damage to roots and shoots. Medium adhered around the roots was removed by washing in running tap water to prevent microbial infection.

The plantlets were then transplanted into plastic pots containing sterile soil, sand and cow dung in a 1:2:1 ratio. The pots were then covered with clear polyethylene bag to maintain high humidity conditions and kept in the growth chamber for proper hardening. Gradually the plantlets were adapted to soil and established. Among the four genotypes, Tori-7 produced highest survived plants (62.25%) followed by BARI Sarisha-9 (60%) (Figure 2).

Conclusions

Leaf segments from six to seven days old seedlings of four *Brassica* varieties were cultured on MS medium supplemented with different concentrations and combinations of hormone. Callus induction was highest in BARI Sarisha-9 (96.11%) and lowest in Sampad genotype (62.78%). The interaction between BARI Sarisha-9 and MS medium supplemented with 1.5 mg L^{-1} 2, 4-D+0.3 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ showed best performance for callus induction with shortest initiation days (6.833 days). Again, callus of BARI Sarisha-9 cultured MS medium supplemented with 3.5 mg L^{-1} BAP+0.5 mg L^{-1} NAA+2.0 mg L^{-1} $AgNO_3$ showed best performance for both shoot

induction and minimum time for shoot initiation. The highest number of root was observed in BARI Sarisha-9 (76.67%) and lowest number was found in Sampad varieties (27.78%). The maximum number of roots was observed in ½ MS medium supplemented with 0.5 mg L^{-1} against all the genotypes. The number of roots decreased with increasing NAA concentrations. Considering the overall performances, we observed that though BARI Sarisha-7 produced slightly higher number of plants than Tori-7 but BARI Sarisha-7 was best genotype for callus, shoot and root induction under *in-vitro* condition. Therefore, future micro-propagation program might be successful with BARI Sarisha-7 variety.

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