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Research Note

Effect of cytokinins on shoot regeneration from cotyledon and leaf segment of stem mustard (*Brassica juncea* var. *tsatsai*)

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Abstract

Cotyledon and leaf segments of stem mustard (*Brassica juncea* var. *tsatsai*) were cultured on Murashige and Skoog medium supplemented with various concentrations of different cytokinins [6-benzyladenine (BA), *N*-(2-chloro-4-pyridyl)-*n*-phenylurea (CPPU), 6-furfurylaminopurine (KT) and thidiazuron (TDZ)] in combinations with different levels of α-naphthalene acetic acid (NAA). The shoot regeneration frequency of cotyledon and leaf segment was dependent on the kinds and concentrations of cytokinins used in the medium, while in most cases cotyledon gave high regeneration frequency than leaf segment. TDZ proved to be the best cytokinin to induce shoot from both cotyledon and leaf segments compared to BA, KT and CPPU. The highest frequency of shoot regeneration was 61.3–67.9 % in cotyledon and 40.7–52.4% in leaf segment respectively when 2.27 or 4.54 μM TDZ was combined with 5.37 μM NAA. Next to TDZ, CPPU was also very suitable to induce shoot formation both in cotyledon and leaf segment. When 1.61 μM CPPU was combined with 2.69 μM NAA, shoot regeneration frequency was 45.0% in cotyledon and 36.4% in leaf segment, respectively. It was also shown that KT and BA affected shoot regeneration from cotyledon and leaf segment, the shoot regeneration was greatly increased when NAA was added together with cytokinins. The efficient and reliable shoot regeneration system was developed in both cotyledon and leaf segments. This regeneration protocol may be applicable to the improvement of this crop by genetic engineering in the future.

Abbreviations: BA – 6-benzyladenine; CPPU – N-(2-chloro-4-pyridyl)*n*-phenylurea; KT – 6-furfurylaminopurine (kinetin); MS – Murashige and Skoog medium; NAA – α-naphthalene acetic acid; TDZ – thidiazuron

Due to the long history of cultivation and selection, *Brassica juncea* has several cultivars which are a rich source of germplasm for future crop improvement. Stem mustard (*B. juncea* var. *tsatsai*), tsatsai in Chinese, is a very important vegetable crop grown in China (Liu, 1996). Stem mustard is a swelling stem vegetable widely grown in the Changjiang River valley, especially in Shichuan and Zhejiang provinces of China, but is not well known in other countries (Guo et al., 1994). Successful *in vitro* culture, regeneration, propagation and transformation from other *Brassica* species have been reported (Mathews et al., 1985, 1990; Metz et al., 1995; Eapen and George, 1997; Cao and Earle, 2003; Wahlroos et al., 2003). However, a systematic study of *in vitro* culture of stem mustard is lack. It is necessary to establish the efficient regeneration

system for mass micropropagation and genetic manipulation in this crop because genotypic difference in shoot regeneration existed in *B. juncea* (Chi et al., 1990; Mathews et al., 1985). In this study, we report the effect of different cytokinins and their concentrations on shoot regeneration of stem mustard and developed a reliable shoot regeneration system by using the most effective cytokinin.

Seeds of stem mustard (*B. juncea* var. *tsatsai* cv. Suotouzhong) were washed under running tap water for 2 h followed by a treatment with 80% ethanol for 1 min, 0.1% $HgCl_2$ for 15 min and then rinsed with sterile distilled water for three to four times. The seeds were then inoculated on the growth regulator free MS (Murashige and Skoog, 1962) medium. After 2-week culture, cotyledon and leaf segments were collected, and cut into small pieces of about 0.5×0.5 cm for further experiments. The MS medium supplemented with 3% (w/v) sucrose was used as a basal medium. The medium was solidified with 0.7% (w/v) agar. Plant growth regulators (BA, CPPU, KT, TDZ and NAA) at various concentrations were added and pH was adjusted to 5.7 before autoclaving at 121 °C for 20 min. Cultures were incubated in flasks (50 ml) containing 20 ml medium and maintained at 25 ± 2 °C under a 12-h photoperiod at a light intensity of 45 μmol m^{-2} s^{-1}. After 4 weeks of culture, the percentage of explants with adventitious shoots out of the total number of explants (%) was scored. About 20 explants were used in each treatment and the experiment was repeated 3 times. All data were subjected to statistical analysis using Duncan's Multiple Range Tests at the 1% level.

Callus was induced from cotyledon explants after 10–12 days of culture on MS media containing different cytokinins (BA, KT, CPPU and TDZ) and auxin NAA. Calli were initiated at the cut ends of the explants and eventually extended all over the explants. Regeneration of green shoot buds occurred in these calli after 3 weeks of culture. The maximum number of shoot bud occurred in the fourth week of culture. More shoots were differentiated at the cut edges than the explant surface. The effect of BA, KT, CPPU and TDZ alone or in combinations with NAA on frequency of shoot regeneration from cotyledon was presented in Table 1. Without addition of auxin NAA, 4.44 μM BA was the

Table 1. Effect of different cytokinins combination with NAA on shoot regeneration (expressed as the percentage of total explants that produced shoots) derived from cotyledon of stem mustard after 4-week culture

NAA (μM)	BA (μM)				KT (μM)				CPPU (μM)				TDZ (μM)			
	0	4.44	8.88	17.76	2.32	4.65	6.97	9.29	0.40	0.81	1.61	3.23	6.46	2.27	4.54	6.81	9.08	11.35
0	0 g	23.4a*	15.1bc	1.3g	5.6efg	12.7cd	11.1cde	5.9efg	7.4def	8.6def	11.8cde	0.2g	0g	14.7bcd	19.4ab	4.8fg	2.1g	4.5fg
2.69		38.9bcd	36.3bcd	35.7bcd	11.1ij	22.5fgh	18.8ghi	16.7ghi	10.5ij	21.1fgh	45.0a	33.3cde	31.8cdef	22.4fgh	26.1efg	25.0efg	18.4ghi	11.1ij
5.37		35.3bc	27.8cd	17.1defg	5.8h	13.7efgh	11.1fgh	8.3gh	24.2cdef	25.3cdef	27.8cd	26.9cd	24.1cdef	61.3a	67.9a	41.2b	34.6bc	27.4cd

*Data within rows followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range test.

optimum concentration for shoot regeneration, the frequency was 23.4%. This was quite similar to the regeneration medium used by Mathews et al. (1990). They cultured cotyledons from 6-day-old seedlings of mustard *B. juncea* (L.) Czern and Coss for shoot regeneration. TDZ at concentration of 4.54 μM was also good for shoot regeneration; the regeneration frequency was 19.4%. With increase in BA or TDZ concentration, shoot induction was greatly inhibited. Without addition of auxin NAA, shoot regeneration frequency from KT or CPPU treatment was significantly lowered compared to BA and TDZ used. Shoots were only induced at lower concentrations of CPPU. Hardly any regenerated shoot was observed in higher concentration of CPPU (≥3.23 μM, Table 1). Shoot induction in all cytokinins used was obviously enhanced in the presence of auxin NAA. When 2.69 μM NAA was added in the medium combined with other cytokinin, 4.44 μM BA, or 1.61 μM CPPU or 4.54 μM TDZ gave the highest shoot regeneration potential. At these combinations, their regeneration frequencies were 38.9, 45.0 and 26.1%, respectively (Table 1). These results are similar to Wahlroos et al (2003). They found that 10.74 μM NAA and 17.76 μM BAP was the most optimal resulting in 41.6% shoot regeneration efficiency of 5-day-old cotyledon explants from *Brassica rapa*. However, in our study, when auxin NAA was increased to 5.37 μM in the medium combined with other cytokinin BA, KT and CPPU respectively, shoot induction was greatly decreased. But in TDZ treatments shoot regeneration were significantly increased when auxin NAA concentration was up to 5.37 μM. At TDZ concentration of 2.27 or 4.54 μM, the highest frequency of shoot regeneration reached 61.3 and 67.9 %, respectively (Figure 1 and Table 1). With increasing concentration of TDZ from 4.54 to 11.35 μM, shoot induction was dramatically decreased to 27.4%.

Figure 1. Shoot was regenerated from cotyledon explants on MS medium with 4.54 μM TDZ and 5.37 μM NAA after culture for 4 weeks.

The effect of BA, KT, CPPU and TDZ alone or in combinations with NAA on shoot regeneration from leaf segment was summarized in Table 2. Shoot induction derived from leaf segment with KT treatments was much better than that of cotyledon. Without addition of auxin NAA, 2.32 μM KT was the optimum concentration for shoot regeneration in leaf segment compared to other cytokinins BA, CPPU and TDZ used, the frequency was up to 22.2% (Table 2). The shoot regeneration frequency from leaf segments was also obviously enhanced in the presence of auxin NAA in all cytokinins used. When 2.69 μM NAA was added in the medium combined with other cytokinin, 4.44 μM BA, 2.32 μM KT, 4.54 μM TDZ and 1.61 μM CPPU gave the highest shoot regeneration potential (Figure 2 and Table 2). At these combinations, regeneration frequencies were 37.4, 37.5, 28.5 and 36.4%, respectively. When auxin NAA was increased to 5.37 μM in the medium combined with other cytokinin BA and CPPU respectively, shoot regeneration was not much different compared to NAA at concentration of 2.69 μM. But in TDZ treatment shoot induction was significantly increased when auxin NAA was up to 5.37 μM. The highest frequency of shoot regeneration was 40.7% and 52.4% when leaf segments were cultured on 2.27 and 4.54 μM TDZ, respectively (Table 2). With increasing concentration of TDZ up to 11.35 μM, shoot induction was also dramatically decreased to 23.8%. So, at the range of TDZ concentration from 2.27 to 4.54 μM combined with 5.37 μM NAA, it was the best combination for shoot regeneration from leaf segment.

Mathews et al. (1985, 1990) reported successful regeneration and transformation from mustard *B. juncea* (L.) Czern and Coss which is an important oilseed crop in India. Our stem mustard (*B. juncea* var. *tsatsai*), which is a swelling stem vegetable widely grown in China, is completely different with above mustard. Based on our knowledge, this is the first study in stem mustard that we systematically compared the

Table 2. Effect of different cytokinins combination with NAA on shoot regeneration (expressed as the percentage of total explants that produced shoots) derived from leaf segment of stem mustard after 4-week culture

NAA (μM)	BA (μM)			KT (μM)			CPPU (μM)				TDZ (μM)				
	0	4.44	8.88	17.76	2.32	4.65	6.97	9.29	0.40	0.81	1.61	3.23	6.46	2.27	4.54	6.81	9.08	11.35
0	0 jk	18.3b	16.8b	14.6b	22.2a*	12.7bcd	12.5bcd	6.7efg	1.0ijk	6.2fghi	8.4cdef	7.7defg	6.4efg	12.5bcd	12.8bcd	8.2cdef	3.1fehij	2.7ghij
2.69		37.4a	33.8bc	31.bc	37.5a	31.6bc	21.4fghi	12.1igk	26.7def	28.6cdef	36.4a	23.1fgh	21.4fghi	20.9fghi	28.5cdef	22.9fghi	16.3hij	15.2ilk
5.37		34.2cdef	31.3cdef	26.5efg	26.1efg	25.2fg	21.7 fg	20.8 fg	20.0fg	21.4fg	31.3cdef	27.5 defg	26.7efg	40.7bc	52.4a	38.1bc	30.5cdef	23.8fg

*Data within rows followed by the same letter are not significantly different at the 1% level by Duncan's Multiple Range Test.

Figure 2. Shoots were induced from leaf segments on MS medium with 4.44 μM BA and 2.69 μM NAA after culture for 4 weeks.

effects of different cytokinins such as BA, KT, CPPU, and TDZ on shoot regeneration from two types of explants, cotyledon versus leaf segment. The results demonstrated that it is possible to directly induce shoot regeneration by using different cytokinins combination with NAA from both cotyledon and leaf segments of *B. juncea* var. *tastsai*. Among the cytokinins tested, TDZ proved to have the highest shoot regeneration frequency from 61.3 to 67.9% in cotyledon and from 40.7 to 52.4 % in leaf segment when 2.27–4.54 μM TDZ was combined with 5.37 μM NAA, respectively. Our results are similar to those of Kim et al. (1997), who reported optimal TDZ for stable shoot proliferation of green ash. Next to TDZ, CPPU was also suitable to induce shoot formation both in cotyledon and leaf segment. KT indicated poor response of shoot formation in cotyledon. But shoot regeneration in leaf segment was much better than that of cotyledon (Table 2). In the present study, higher concentration of NAA suppressed the rate of shoot regeneration in some cases, but shoot morphology was not affected. In general, the explant type had a different regenerative capacity; cotyledon in most cases had high shoot regeneration frequency and regenerated much more shoots than leaf segment.

The results show that a high frequency of shoot regeneration in *B. juncea* var. *tsatsai* could be achieved both from cotyledon and leaf segments. The protocol reported here is efficient and reliable. Just like other *Brassica* species (Mathews et al., 1990; Metz et al., 1995; Cao and Earle, 2003;

Wahlroos et al., 2003), which were reported in successful genetic transformation, this protocol may be useful in genetic improvement by using transgenic approach because this crop is suffering a severe virus infection in China.

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