

Full Length Research Paper

Optimization of a plant regeneration protocol for broccoli

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The factors which influence the regeneration of broccoli (*Brassica oleracea* var. *italica*) were studied using an orthogonal design. The results showed that the major factor was the explant type, followed by naphthylacetic acid (NAA), benzylaminopurine (BAP), sucrose and AgNO₃ in turn. The maximum regeneration was on Murashige and Skoog (MS) medium + NAA 0.107 μM + BAP 17.76 μM + 2% sucrose + 0.8% agar. Hypocotyl proved to be the optimum explant source and its regeneration frequency reached 100%.

Key words: Naphthylacetic acid (NAA), benzylaminopurine (BAP), AgNO₃, hypocotyl, broccoli (*Brassica oleracea* var. *italica*).

INTRODUCTION

Broccoli (*Brassica oleracea* L. var. *italica* Plenck), which originated from Italy and is related to the cabbage and cauliflower, is a very important vegetable crop. It is well known for its high vitamin, calcium and sulforaphane content (Zhang et al., 1992; Henzi et al., 2000). Plant diseases, insects and other stressors can cause enormous yield reductions during commercial broccoli cultivation (Yang et al., 2002; Cao and Earle, 2003; Viswakarma et al., 2004) but, under modern production systems, genetic engineering can be used to add target characteristics to broccoli cultivars. Transgenic *B. oleracea* plants have been obtained using several methods involving *Agrobacterium*-mediated transformation (Boulter et al., 1990; Toriyama et al., 1991; Berthomieu and Jouanin, 1992; Christey and Sinclair, 1992; Metz et al., 1995; Christey et al., 1997; Higgins et al., 2006; Jocelyn et al., 2007). The efficiency of such *Agrobacterium*-

mediated transformation is known to be affected by multiple factors such as the bacterial strain and concentration used, the plant genotype, explant type and co-cultural conditions (van Wordragen and Dons, 1992; Puddephat et al., 1996; Birch 1997; Cogan et al., 2002; Chakrabarty et al., 2002; Kim and Botellam, 2002; Nigel et al., 2002; Suri et al., 2005). Most authors have reported difficulties with one or more of the stages in the gene transfer and regeneration process which prevent any of their protocols from serving as the method of choice for *B. oleracea* transformation. Transformation efficiency can however, be increased by manipulating many factors, and the important factors are the explant and the plant growth regulator.

There have been many reports on plant regeneration of Brassica crops, but they studied the factors that affect plant regeneration separately, such as explant type (Dai et al., 2009), plant regulator concentration (Dai et al., 2009; Ravanfar et al., 2009) and sucrose concentration. This study reports the optimization of a system for broccoli regeneration by orthogonal design. The improved regeneration was achieved by systematically optimizing the combination of factors that affect plant regeneration using an orthogonal design (Tang and Feng, 1997) in *Catharanthus Roseus* (Sun et al., 2002), white clover (Zhang et al., 1998) and so on. Together,

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Abbreviations: MS, Murashige and Skoog; BAP, benzylaminopurine; NAA, naphthylacetic acid.

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Table 1. Orthogonal design table.

Level code	NAA (μM)	BAP (μM)	AgNO ₃ (mM)	Sucrose (%)	Explant
1	0	0	0	1	Cotyledon
2	0.054	8.88	0.02	2	Cotyledon plus petiole
3	0.107	17.76	0.04	3	Petiole
4	0.161	26.64	0.06	4	Hypocotyl

Table 2. Broccoli regeneration frequency results*.

Medium no.	NAA (μM)	BAP (μM)	AgNO ₃ (mM)	Sucrose (%)	Explant	Mean number of shoots per explant	Regeneration frequency (%)
1	1(0)	1(0)	1(0)	1(1)	1 (Cotyledon)	1.4±0.54	6±1.58 ^K
2	1(0)	2(8.88)	2(0.02)	2(2)	2 (Cotyledon plus petiole)	2.0±0.71	22±3.53 ^F
3	1(0)	3(17.76)	3(0.04)	3(3)	3 (Petiole)	1.6±0.54	9.33±1.78 ^U
4	1(0)	4(26.64)	4(0.06)	4(4)	4 (Hypocotyl)	5.8±1.48	92.22±0.84 ^B
5	2(0.054)	1(0)	2(0.02)	3(3)	4 (Hypocotyl)	3.6±0.89	72.89±2.28 ^D
6	2(0.054)	2(8.88)	1(0)	4(4)	3 (Petiole)	2.0±1.22	13.78±1.10 ^H
7	2(0.054)	3(17.76)	4(0.06)	1(1)	2 (Cotyledon plus petiole)	1.6±0.89	8.22±1.30 ^{JK}
8	2(0.054)	4(26.64)	3(0.04)	2(2)	1 (Cotyledon)	0.0±0.00	0±0.00 ^L
9	3(0.107)	1(0)	3(0.04)	4(4)	2 (Cotyledon plus petiole)	2.0±1.00	9.11±1.22 ^U
10	3(0.107)	2(8.88)	4(0.06)	3(3)	1 (Cotyledon)	2.2±1.30	11.78±1.48 ^{HI}
11	3(0.107)	3(17.76)	1(0)	2(2)	4 (Hypocotyl)	6.4±1.14	100±0.00 ^A
12	3(0.107)	4(26.64)	2(0.02)	1(1)	3 (Petiole)	1.8±1.10	8.67±1.92 ^{JK}
13	4(0.161)	1(0)	4(0.06)	2(2)	3 (Petiole)	2.4±0.89	16.89 ±2.59 ^G
14	4(0.161)	2(8.88)	3(0.04)	1(1)	4 (Hypocotyl)	3.8±0.84	86.67±0.89 ^C
15	4(0.161)	3(17.76)	2(0.02)	4(4)	1 (cotyledon)	2.4±1.14	8.89±1.64 ^{JK}
16	4(0.161)	4(26.64)	1(0)	3(3)	2 (Cotyledon plus petiole)	2.8±1.30	28.89±1.87 ^E

*The regeneration frequency was recorded 21 d after inoculated, 450 explants were used for the experiment, A-F expresses differences at the level of $\alpha=0.01$ level.

these modifications resulted in a notable improvement in the regeneration rate of the species.

MATERIALS AND METHODS

Broccoli (*B. oleracea* L. var. *italica* Plenck) cv Xinlv was provided by the Jiangsu Academy of Agricultural Sciences, Nanjing. Seeds, surface sterilized in 70% ethanol for 90 s, 0.1% HgCl₂ for 12 min, were germinated at 25°C on solid MS medium (Murashige and Skoog, 1962) containing 2% sucrose (w/v) (pH 5.8). A 16 h photoperiod was used.

Medium constituents and culture conditions

All media used in this experiment were of MS type with 0.8% agar plus different concentrations of benzylaminopurine (BAP: 0, 8.88, 17.76 and 26.64 μM) and naphthylacetic acid (NAA: 0, 0.054, 0.107 and 0.161 μM). The medium with a pH adjusted to 5.8 was dispensed into 100 ml Erlenmeyer flasks before autoclaving at 121°C and 1.2 ~ 1.3 kgcm⁻² for 20 min, and silver nitrate (AgNO₃: 0, 0.02, 0.04 and 0.06 mM) was added after being filter sterilized. The medium was dispensed into 11 cm Petri dishes. The culture conditions were 25 ± 1°C and a 16 h photoperiod of approximately

28 $\mu\text{Em}^{-2} \text{s}^{-1}$. The rooting medium was composed of MS + 0.107 μM NAA + 2% sucrose + 0.8% agar, at pH 5.8.

Shoot and root induction

Cotyledon, cotyledon with petiole attached, petiole and hypocotyl explants were isolated from 7 days old germinated seedlings and cultured on regeneration medium for adventitious bud induction (Table 1), the orthogonal combination scheme is provided in Table 2. Five replications and 90 explants per replicate was used for each treatment, and data variance analysis by DPS was used (Tang et al., 1997).

Explants were inoculated on differentiation regeneration media for adventitious bud induction. Adventitious buds were then cut and transplanted onto root induction media. The root induction medium composed of MS + NAA (0, 0.54 and 1.07 μM) + 3% sucrose + 0.8% agar, and the regenerated plants were transplanted onto vermiculite medium after 2 weeks of root induction.

RESULTS

By examining the combination of factors that affected broccoli regeneration using an orthogonal design and by

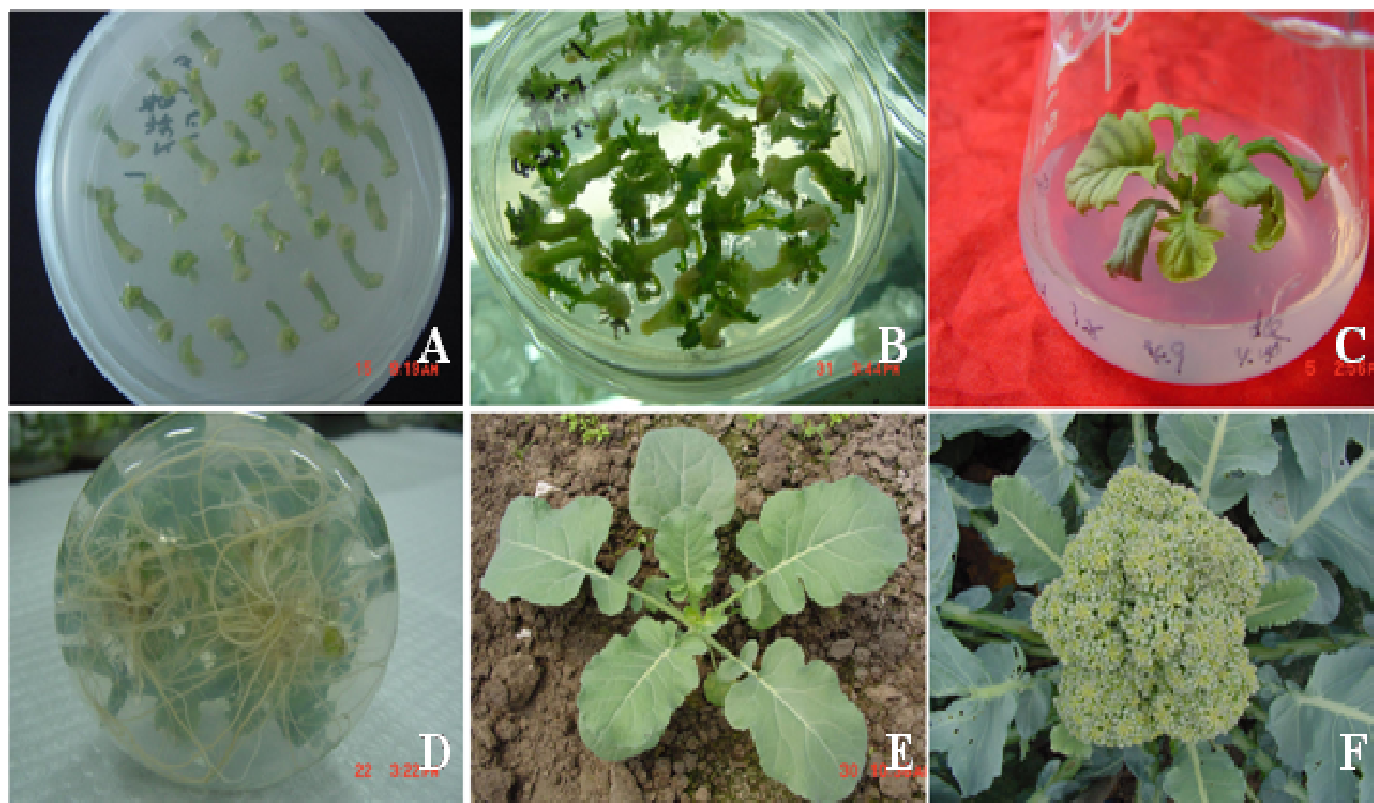


Figure 1. The regeneration of broccoli hypocotyle from cv 'Xinlv' (*B. oleracea* var. *italica* cv 'Xinlv'). A: Calli formation (7 days); B: formation of the regenerated bud (21 days); C: further growth of a regenerated bud (42 days); D: root formation in the regenerant (56 days); E: The transplantation of a regenerated broccoli seedling (90 days); F: flower head formation (150 days).

the use of data variance analysis by DPS (Tang et al. 1997), it was apparent that the most suitable condition for Xinlv was: MS + NAA 0.107 μ M + BAP 17.76 μ M + 2% sucrose + 0.8% agar. Hypocotyls were the best explant source ($F_{\text{explants}} = 1161.897$, $F_{0.01} = 5.29$) with a regeneration frequency of 100% (Table 2 and Figure 1).

The effect of plant growth regulator on broccoli regeneration

The plant growth regulator kind and concentration have major effect on the broccoli callus induction and differentiation. The results indicated that the different NAA and BAP level have highly significant difference on broccoli regeneration ($F_{\text{NAA}} = 18.8723$, $F_{\text{BAP}} = 8.3232$, $F_{0.01} = 5.29$), and 0.107 μ M NAA, 17.76 μ M BAP is the best for the broccoli regeneration.

The effect of silver nitrate on broccoli regeneration

Broccoli regeneration is not sensitive to silver nitrate, the broccoli adventitious bud regenerated better while there is no silver nitrate in the medium than the

medium with silver nitrate. So for the broccoli regeneration medium, the silver nitrate should not be added.

The effect of sucrose concentration on broccoli regeneration

There is a highly significant difference level of different sucrose concentration on broccoli regeneration ($F_{\text{sucrose}} = 6.45344$, $F_{0.01} = 5.29$), and the 2% sucrose is the best concentration for broccoli regeneration.

The effect of explant type on broccoli regeneration

We selected the cotyledon plus petiole, hypocotyls, cotyledon and petiole as the adventitious bud induction explants. The different explant type has a highly significant difference in the broccoli adventitious bud induction ($F_{\text{explant}} = 1081.924$, $F_{0.01} = 5.29$).

Regenerated buds were placed on rooting medium and that which proved optimum for rooting was MS + 1.07 μ M NAA + 3% sucrose + 0.8% agar with a rooting success rate of 100%. All roots produced on this media were rosettes, whilst buds failed to develop on any other media.

DISCUSSION

The establishment of a regeneration system is a major step in the development of transgenic technology for *Brassica* vegetables. This paper defined such a system for *B. oleracea* L. var. *italica* Plenck using selection based on the optimum explants type, plant growth regulators, sucrose and AgNO₃ concentration which proved capable of achieving 100% regeneration from hypocotyl. The kind and concentration of plant growth regulators, sucrose concentration and the explant type all affected the regeneration of broccoli, while AgNO₃ also had some influence on the success of regeneration percentage. The experiment validates the conclusions reached using other broccoli varieties in which the regeneration frequency was also 100% and suggests that the regeneration system is applicable to a range of broccoli varieties (Figure 1). The kind and concentration of plant growth regulators also have a strong effect on broccoli callus induction and differentiation (He et al., 1998; Zhang and Gong, 2001; Zhao et al., 2002; Ravanfar et al., 2009; Dai et al., 2009; Ravanfar et al., 2009). Significant effects of NAA and BAP levels on regeneration ($F_{\text{NAA}} = 23.2379$, $F_{\text{BAP}} = 10.0031$, $F_{0.01} = 5.29$) indicate that both affect regeneration frequencies. Ravanfar et al., (2009) indicated that 96.67% of hypocotyl explant produced shoot on 3 mg·L⁻¹ (13.32 μM) BAP, this study has presented 100% regeneration at 4 mg·L⁻¹ (17.76 μM), because there is a combination relationship among the NAA, BAP, AgNO₃ and the explants. We studied the factors systematically, and the results of regeneration frequency (100%) and mean number of shoots per explant (6.4) indicated that this is the best recipe for broccoli regeneration in this paper.

The results indicated that the medium lacking AgNO₃ was optimum for broccoli regeneration ($F_{\text{AgNO}_3} = 22.1231$, $F_{0.01} = 5.29$). It may therefore be appropriate to tailor the AgNO₃ concentration in the medium according to the plant genotype (Chi et al., 1990). The sucrose concentration was found to significantly affect the degree of regeneration ($F_{\text{sucrose}} = 8.1219$, $F_{0.01} = 5.29$). Several reports have described variety-specific differences in *in vitro* organogeny or embryogenic development in response to contrasting sucrose concentrations (He et al., 1998; Kantia and Kothari, 2002; Tang et al., 2002). Sucrose concentration has an effect on adventitious bud induction and the amount of cluster buds. The differentiation ability and plant growth regulator level of explants depend upon the position and timing of excision, which also affect the regeneration frequency (Murata and Orton, 1987). Hypo-cotyls, cotyledons, petioles and cotyledons in combination with their petioles have been used as explants in this study. Some of these explants have a high regeneration frequency and are easily infected by Agro-bacterium, making them good transform explants. Cotyledons plus petioles are usually chosen as explants during *Brassica* regeneration because, when cut from a growth point, they have some primordial meriste-

matic cells and possess higher regeneration frequencies (Birch, 1997; Kim and Botellam, 2002; Murata and Orton, 1987; Puddephant et al., 1996). It was apparent that hypocotyls were the best explants for broccoli regeneration in this study as they exhibited higher differentiation ability and the plant growth regulator combination was suitable for bud induction. It has been proposed that polarity occurs during *Brassica* hypocotyl regeneration but, in agreement with Li and Chen (1993; 1994), this was not observed in the present experiment using *B. oleracea* L. var. *italica* Plenck.

Conclusions

The best regeneration recipe for the broccoli is on medium MS + NAA 0.107 μM + BAP 17.76 μM + 2% sucrose + 0.8% agar, and the best explant source is hypocotyl. Based on this menu, the regeneration frequency of broccoli reached 100%.

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