Nutrition And Food Science Journal

SCIENTIFIC LITERATURE

Special Issue Article "Broccoli"

Short Communication

Determination of Minimum Inhibitory Concentration of Kanamycin and Dot Blot Analysis for Effective Screening of Transgenic Broccoli Plants

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ARTICLE INFO

Received Date: April 18, 2022
Accepted Date: May 10, 2022
Published Date: May 13, 2022

KEYWORDS

Brassica oleracea
Dot blot analysis
Kanamycin
Minimum inhibitory concentration
MIC
Transgenic plants

Citation for this article: Seyed Ali Ravanfar, Maheran Abd Aziz and Samira Samarfard. Determination of Minimum Inhibitory Concentration of Kanamycin and Dot Blot Analysis for Effective Screening of Transgenic Broccoli Plants. Nutrition And Food Science Journal. 2022; 5(1):133

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ABSTRACT

A Minimum Inhibitory Concentration (MIC) of an antibiotic is needed to separate transformed cells from the non-transformed. In this study, pGreen0049 binary vector which was used in the genetic transformation of broccoli cv. Green Marvel for heat tolerance contains kanamaycin resistance gene as the plant selectable marker gene. Results showed that the highest explant mortality rate occurred on medium containing 60 mg/l kanamaycin on hypocotyl and 90 mg/l on shoot tip explants. Meanwhile, in the control (0 mg/l kanamycin) 93.33 % of the hypocotyl and 96.67 % of shoot tip explants regenerated shoots and produced the highest percentage of regeneration. The results also showed that treatments containing 60 and 90 mg/l kanamycin in hypocotyl and shoo tip explants respectively produced zero mean number of shoots on survived explants. Economically, 50 mg/l kanamaycin for hypocotyl and 80 mg/l kanamycin for shoot tip was therefore selected as the minimum inhibitory concentration for effective screening of putative transformants from hypocotyl explants of broccoli cv. Green Marvel following *Agrobacterium*-mediated transformation. After screening of putative transformants through the MIC test, the dot blot assay is one method performed for detecting the presence of the *AtHSP*101 cDNA in the genome of the broccoli plants. Result showed positive signals with the CSPD substrate (chemiluminescent alkaline phosphatase substrate) for the transgenic lines (1, 2, 3 and 4) however, it was not detectable for the non-transformed control plant. Dot blot assay confirmed the presence of the gene in the transgenic broccoli plants before further confirmation through southern blot analysis.

INTRODUCTION

Broccoli, *Brassica oleracea* subsp. *italica*, is a universally important vegetable. It is a member of the cabbage family, and is intimately related to cauliflower. Broccoli has many health benefits. It could prevent or heal illnesses, as well as breast, colon, lung, bladder, ovarian and prostate cancers, and stomach tumors [1]. TDZ (Thidiazuron), played a critical role in promoting shoot differentiation from different crops. It has been shown to stimulate adventitious shoot regeneration in a large number of plant species such as strawberry, broccoli, cabbage and tomato [1,2]. The presence of an

antibiotic resistance gene as a marker gene in a binary vector is supposed to increase the antibiotic resistance characteristic of transformed plants [3]. There are several antibiotic resistance genes which are commonly applied in the construction of a transformation vector for selection of plant transformants such as kanamycin, streptomycin, bleomycin and hygromycin resistance genes. Among the most widely applied selectable markers are the kanamaycin and hygromycin resistance genes [4,5]. Before transformation, the Minimum Inhibitory Concentration (MIC) of the antibiotic must be determined in non-transformed plants to know the extent of resistance (Andrews 2004) [3]. In genetic transformation of cabbage cv. KY Cross [6], hygromycin resistance gene was the marker gene available inside the vector construct. The MIC of hygromicin determined at 3.5 mg/l for hypocotyl and 10 mg/l for shoot tip allowed the selection of cabbage putative transformants from the non putatives before subsequent molecular assays. Meanwhile [7], found 20mg/l kanamaycin as the best concentration for screening of putative transgenic Indian mustard plantlets. In dot blot assay, the biomolecules to be detected are not separated by electrophoresis. Instead, a mixture containing the molecule to be detected is applied directly on a membrane as a dot, and then is spotted through circular templates directly onto the membrane or paper substrate [8]. Dot blot can be applied either for DNA, RNA or protein by direct spotting to a membrane with no requirement of electrophoretic separation [9]. Oikawa et al. [10] established a simple, efficient and reliable dot-blot technique for *B. rapa* and *B. oleracea* using allele-specific oligonucleotide probes and allele-specific primer pairs designed from sequences of each SP11 allele. Zamani, [11] reported effective screening of transgenic canola cotyledonary petioles by genomic DNA dot blotting. This paper reports on the determination of MIC of kanamycin prior to the transformation of broccoli cv. Green Marvel for heat tolerance using pGreen0049 binary vector which carries the kanamaycin resistance gene as the plant selectable marker. Following transformation, dot bot analysis was performed as one of the best methods to confirm the integration of the transgene into the plant genome and quickly detection of plant events before southern blotting.

MATERIALS AND METHOD

The seeds of broccoli cv. Green Marvel were placed under running tap water for 15 min followed by immersion and shaking in 70 % ethanol for 1 min. The seeds were rinsed once with sterile distilled water followed by immersion and shaking in 1.05 % sodium hypochlorite (NaOCl) with 1-2 drop(s) Tween 20 for 10 min. Finally the seeds were rinsed with sterile distilled water three times and cultured on a germination medium consisting of half-strength MS [12] salts supplemented with 2.8 g/lphytagel and 30 g/l sucrose. The sterilization of seeds was carried out according to Ravanfar et al. [13] with some modifications. Hypocotyl segments, 5-8 mm in size and shoot tips, 8 mm in size, were isolated from 6-day-old seedlings and used for determining the MIC of kanamycin for both explants.

The hypocotyl explants were cultured on Shoot Regeneration Medium (SRM) consisting of MS salts and vitamins, 30 g/L sucrose, 0.1 mg/l TDZ 2.8 g/L phytagel and supplemented with nine different concentrations of kanamycin (0, 10, 12, 15, 20, 30, 40, 50 and 60 mg/l). The shoot tip explants were cultured on Shoot Regeneration Medium (SRM) containing MS salts and vitamins, 30 g/L sucrose, 1.5 mg/l zeatin, 2.8 g/L phytagel and supplemented with 10 different concentrations of kanamycin (0, 10, 20, 30, 40, 60, 70, 80, 90 and 100 mg/l). Parameters recorded were the percentage of regeneration from survived explants and mean number of shoots per survived explant. Survived explant refers to an explant that remained green.

The experiments were arranged in a Completely Randomized Design (CRD). Each treatment was replicated three times and each replication per treatment contained 10 explants. Data were analysed using the Analysis of Variance (ANOVA). Duncan New Multiple Range Test (DNMRT) at α= 5% was used for comparison between treatment means.

DNA probes were regenerated with DIG-High Prime according to the random primed labelling technique. PCR product fragments, forward primer: 5' GGCGAGGGTAAAGTCTGAGG 3' and reverse primer: 5' GATTCGCACACCATGATGTCC 3' extracted from 1% agarose gel were used to prepare the probes for dot blot hybridization (primers reference should be added here). The probes contained a common region to HSP 101 cDNA. The extracted

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genomic DNA (20 µg) from the fresh leaves of the putative transgenic and non-transformed control plants, was denatured for 10 min in boiling water and chilled on ice. The denatured genomic DNAs were spotted on a nylon membrane (Hybond N+, Amersham) and hybridized to Dig- dUTP labeled HSP101probe. The membrane was incubated in 20 ml antibody solution containing 150 U/ml Anti- digoxingenin-Ap for binding to the DIG-labeled probe. After that the membrane was washed for 15 min two times with washing buffer to remove unbound antibody and incubated for 15 min in the detection buffer. The faced up DNA membrane was covered with 1 ml disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.13,7]decan}-4-yl) phenyl phosphate (CSPD) (substrates for alkaline phosphatase) and incubated for 10 min at 37°C. CSPD is especially suited for highly sensitive and fast detection of non-radioactively-labeled nucleic acids.After 1-5 min at 25°C the blue bands (chemiluminescence) were captured using a Canon camera in the dark room.

RESULTS AND DISCUSSION

Table 1 shows the percentage of regeneration from survived hypocotyl explants at 8 weeks of culture on SRM supplemented with different concentrations of kanamycin. In the first week of culture both ends of the hypocotyl explant began to expand in all kanamaycin treatments. From the fourth to the eighth week of culture, it was observed that the highest mortality rate occurred on medium containing 60 mg/l kanamaycin. None of the survived hypocotyl explants cultured on 60 mg/l kanamaycin produced shoots. Meanwhile, in the control treatment (0 mg/l kanamycin) 93.33% of the explants regenerated shoots and differed significantly from the rest of the treatments (Table1).

Table 1: Percentage of regeneration from survived hypocotyl explants of broccoli cv. Green Marvel after 8 weeks of culture on SRM supplemented with different concentrations of kanamycin.

Kanamycin (mg/l)	Percentage of regeneration
0 (control)	93.33 a
10	70.00 b
12	70.00 b
15	63.33 bc
20	46.67 c
30	26.66 d
40	13.33 de
50	3.33 de
60	0.00 e

Means followed by the same letter (s) are not significantly different based on DMNRT (p=0.05).

Table 2: Mean number of shoots from survived hypocotyl explants of broccoli cv. Green Marvel after 8 weeks of culture on SRM supplemented with different concentrations of kanamycin.

Kanamycin (mg/l)	Mean number of shoots
0 (control)	4.33 a
10	0.90 b
12	0.67 bc
15	0.63 bc
20	0.53 bc
30	0.37 cd
40	0.26 cd
50	0.03 d
60	0.00 d

Means followed by the same letter (s) are not significantly different based on DMNRT (p=0.05).

Table 2 shows treatment containing 60 and 50 mg/l kanamycin produced among the lowest mean number of shoots (0 and 0.03 respectively) on survived hypocotyl explants compared with other treatments. Those treatments showed significant difference on mean shoot number produced compared to treatments containing 0, 10, 12, 15, and 20 mg/l kanamaycin except with 30 and 40 mg/l kanamaycin. Economically, 50 mg/l kanamycin was therefore selected as the minimum inhibitory concentration for effective screening of putative transformants from hypocotyl explants of broccoli cv. Green Marvel following Agrobacterium transformation. (Figure 1A ,B)

Based on Table 3 significant effect of different concentrations of kanamycin was observed on percentage of regeneration from survived shoot tip explants at eight weeks of culture. Kanamycin from 10 to 80 mg/l significantly decreased the regeneration compared to the control (without kanamycin). Among the highest percentage of regeneration from survived shoot tip explants (96.67%) was in the control which differed significantly compared to other treatments except with 10 mg/l and 20 mg/l kanamaycin (Table 3). However maximum death (100%) occurred on 90 and 100 mg/l kanamycin, respectively. After four weeks of culture on SRM supplemented with different concentrations of kanamycin the shoot tip explants swelled in all treatments. By the eighth week, significant difference in mean number of shoots produced was observed between the kanamaycin treatments (Table 4). Among the

lowest mean number of shoots produced from the survived explants were on 100 and 90 mg/l (0.00%), respectively. Treatments without and with low concentrations of kanamaycin (0, 10 and 20 mg/l) produced same shoots from the survived shoot tip explants at eight weeks of culture (Table 4). On medium supplemented with high concentration of kanamycin (90 and 100 mg/l) there was no shoot regeneration with increased in explant death (Table 4) (Figure 1C,D,E,F).

Table 3: Percentage of regeneration from survived shoot tip explants of broccoli cv. Green Marvel after 8 weeks of culture on SRM supplemented with different concentrations of kanamycin.

Kanamycin (mg/l)	Percentage of regeneration
0 (control)	96.67 a
10	93.33 a
20	86.67 ab
30	76.67 b
40	53.33 c
60	46.60 c
70	20.00 d
80	3.33 e
90	0.00 e
100	0.00 e

Means followed by the same letter (s) are not significantly different based on DMNRT (p=0.05).

Table 4: Mean number of shoots from survived shoot tip explants of broccoli cv. Green Marvel after 8 weeks of culture on SRM supplemented with different concentrations of kanamycin.

Kanamycin (mg/l)	Mean number of shoots
0 (control)	3.93 a
10	1.77 b
20	1.67 c
30	1 cd
40	0.80 d
60	0.30 e
70	0.17 e
80	0.03 e
90	0.00 e
100	0.00 e

Means followed by the same letter (s) are not significantly different based on DMNRT (p=0.05).

To confirm the presence of the T-DNA sequence in the transformed regenerated plants, dot blot analysis was performed with specific primer from partial HSP (690 bp) as probe. The result showed chemiluminescence signals in the different transgenic lines which confirmed the integration of the T-DNA sequence in the regenerated transgenic broccoli genome. The control did not show the chemiluminescence signal. Result showed positive signals with the CSPD substrate for the transgenic lines however, it was not detectable in the non-transformed control plant (Figure 2). Figure 2 shows hybridization dots of *AtHSP* 101 in both the hypocotyl and shoot tip derived transformants. No hybridization signal was detected in the control and lines 5, 6, 7 and 8. Among the eight hypocotyl and shoot tip derived transformants confirmed by PCR, four (50 %) showed hybridization in dot blot analysis. The study showed that shoot regeneration from hypocotyl explants decreased with increasing kanamaycin concentration. On SRM medium without kanamaycin as control, maximum percentage and mean number of shoots were regenerated from the hypocotyl explants (93.33 and 4.33) respectively. Increasing the concentration of kanamaycin gradually increased death of the hypocotyl explants.

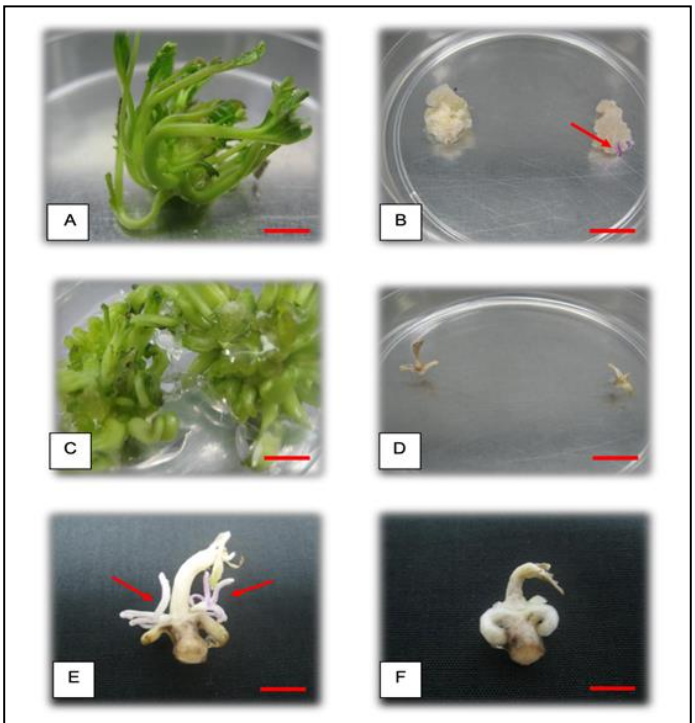

Figure 1: Explants mortality rate on SRM supplemented with different concentrations of kanamycin. (A) 0 mg/l kanamycin (hypocotyl), (B) 50 mg/l kanamycin (hypocotyl), (C) 0 mg/l kanamycin (shoot tip), (D) 90 mg/l kanamaycin (shoot tip), (E) 60 mg/l kanamaycin (shoot tip) and (F) 80 mg/l kanamaycin (shoot tip). Red arrow= Purple shoots. (A), (C), (E) and (F) bar= 5mm, (B) and (D) bar = 10mm.

In medium containing 30 mg/l kanamycin and above, survived hypocotyl explants swelled and produced purple shoots after 4 weeks of culture due to anthocyanins production and loss of chlorophyll activity. At 50 mg/l kanamaycin and higher, the purple shoots were not able to grow and elongate and died after eight weeks of culture. Meanwhile, in medium containing 60 mg/l kanamaycin, some hypocotyl explants survived (remain green) but did not produce any shoots and some became whitish after eight weeks of culture. Similarly, [14] reported that high concentrations of kanamycin bleached almost 100% of the Lycopersicon esculentum cotyledon explants due to loss of chlorophyll which resulted in cell death of the non-transformed explants.

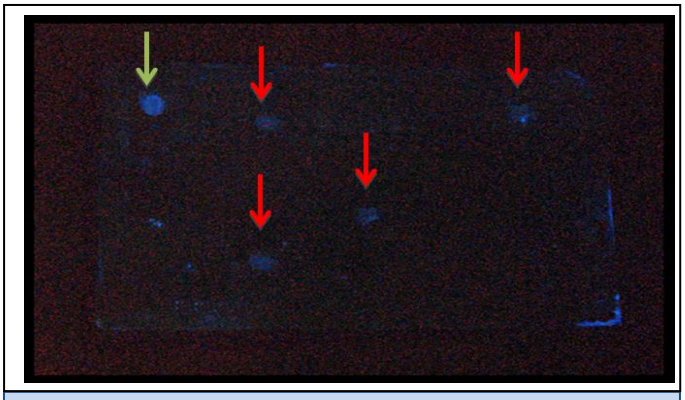

Figure 2: *AtHSP* 101 dot blot assay of leaf samples from kanamycin-selected plants of broccoli cv. Green Marvel. Green Arrow= probe of binary vector pGreen 0049 (positive control), red arrow= hypocotyl and shoot tip-derived transformants.

According to Rafat et al. [6] if any antibiotic resistance gene as a plant selectable marker is available inside a vector construct, the MIC of the antibiotic has to be determined before transformation to allow the selection of putative transformants from the non putatives for subsequent molecular assays. There are several antibiotic resistance genes which are commonly applied in the construction of a transformation vector for selection of plant transformants such as kanamycin, streptomycin, bleomycin and hygromycin resistance genes. Among the most widely applied as selectable markers are kanamaycin and hygromycin antibiotic resistance genes [4].

In this study, the medium without kanamaycin (0 mg/l) produced the highest percentage and mean number of survived shoot tip explants. Medium with 10 to 40 mg/l kanamycin also produced shoots but with decreasing percentage and mean number of shoots (Table 3,4). In medium with 60 mg/l kanamycin, shoots occurred with some purple and whitish color and some of the explants produced callus.

Medium containing 80 mg/l kanamycin resulted in the production of few shoots (1 or 2) but their growth was inhibited by the high concentration of kanamycin after eight weeks of culture. At 90 mg/l kanamycin and above, explants became whitish and died without any shoot formation after eight weeks of culture. According to [14] kanamycin showed severe toxicity effects such as browning and death of *L. esculentum* explants within 4 weeks on selective media which contained higher than 100 mg/l of the antibiotic.

Kanamycin has also been used as a selectable marker in genetic transformation of several *Brassica* species. Li et al. [15] used 15 mg/l kanamycin to select transformed shoots from cotyledon explants of *B. napus*, while Block et al. [16] and [17] applied 50 mg/l kanamycin for the selection of putative transformants from *B. oleracea* hypocotyl explants. Meanwhile Zhao et al. [18] selected putatively transformed shoots of mustard (*B. juncea Coss*) by using 30 mg/l kanamycin. In this study, the higher ability of shoot tip explants of *B. olearcea* cv. Green Marvel to withstand high concentration of kanamycin could be due to the fact that the cells in the shoot tip are more tolerant to kanamycin toxicity because of the continuous cell division process that occurs in that region compared to the hypocotyl cells.

*AtHSP*101 gene had been stably integrated into the genome of four transgenic broccoli plants. Stable integration of transgene into the genome of other *Brassica* species has been demonstrated by dot blot analysis such as in *B. oleracea* var. *botrytis* (cauliflower) [19], *B. oleracea* (Chinese cabbage) [20] and *B. napus* (rapeseed) [21,22]. In this study, some putative transformants confirmed by MIC to be transgenic did not show the hybridization in dot blot analysis probably due to incomplete digestion, hybridization and integration of the *AtHSP*101 gene or occurrence of shoot escapes or methylation into regulatory sequences. According to Rafat et al. [6] the number of escaped shoots could be reduced by increasing the concentration of kanamycin in the selection medium but high kanamaycin concentration may prevent the regeneration of more transformed cells.

From the results of this study 50 mg/l and 80 mg/l kanamaycin were selected as the minimum inhibitory concentrations for hypocotyl and shoot tip explants, respectively. High concentrations of kanamycin stimulated callus formation and promoted formation of some purple shoots on both hypocotyl and shoot tip explants of broccoli cv. Green Marvel. The result also showed increasing concentrations of kanamaycin resulted in gradual increase in death of the hypocotyl and shoot tip explants. DNA dot blot analysis of plants derived of the transgenic broccoli lines showed hybridization signal with probe against the *AtHSP*101 and confirmed the trangenicity of broccoli plants prior to greenhouse and abiotic stress analyses.

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