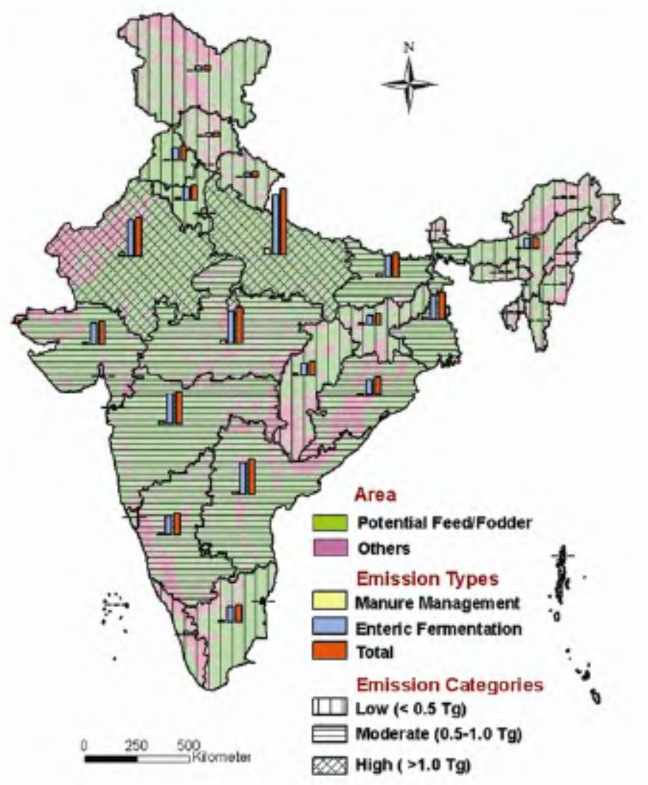

Figure 4. Spatial distribution of methane emission from livestock among States/Union Territories for 2003. (Potential feed/fodder area implies both area under crops and grasslands available for livestock. Other areas include land-cover classes like forest, snow or ice, water bodies, wetlands, bare soil, urban built-up land, etc.)

available for the livestock. Detailed spatial analysis indicated the majority of districts with less than 100 kg/ha methane flux. Few districts in Bihar, Haryana, Karnataka, Kerala, Punjab, Uttar Pradesh and West Bengal reported methane flux in the range 101–250 kg/ha. These outputs of methane emission inventory from the Indian livestock are important inputs for generating spatial integrated multi-source methane emission inventory at the national level.

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Molecular diagnosis of transgenic tomato with *osmotin* gene using multiplex polymerase chain reaction

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The requirement for upgradation of analytical methods for the detection of genetically modified (GM) crops is increasing at a fast pace, with a quantum jump in the area of GM crops being grown globally to meet the regulatory and international trade requirements. In the present study, standardization of multiplex polymerase chain reaction (MPCR) for the detection of

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GM tomato with *osmotin* gene was undertaken. In the MPCR, two pairs of designed primers specific for the inserted *osmotin* gene from *Nicotiana tabacum* in the transgenic tomato were used along with primers for 35S-promoter from *Cauliflower Mosaic Virus* and *LAT 52* (endogenous *late anther tomato*) gene for simultaneous detection of the *osmotin* transgene, 35S promoter and endogenous *LAT 52* gene. Internal control target (*LAT 52* gene) was included both to assess the efficiency of all PCR reactions and to eliminate any false negatives. Molecular analysis using MPCR of the GM tomato revealed the detection of *osmotin* gene, *CaMV* 35S promoter and endogenous *LAT 52* gene simultaneously in a single amplification reaction comprising 35 cycles with 59°C annealing temperature. MPCR detection results of GM and non-GM tomato suggest that the reported MPCR protocols would be suitable for detection and monitoring of transgenic tomato with *osmotin* gene.

Keywords: Genetically modified tomato, molecular diagnosis, multiplex PCR, *osmotin* gene.

TOMATO (*Lycopersicon esculentum* L.), belonging to the family Solanaceae, is an economically important crop in many countries, including India. Genetically modified (GM) tomato has been approved for commercialization in many countries since the first GM tomato 'Flavr Savr' was permitted for planting on large scale in 1994. In 2008, the global area of biotech crops continued to soar and showed an increase of 12% (10.7 m ha) between 2007 and 2008, reaching 125 m ha (ref. 1). With the development and commercialization of GM crops, the need for detection of GM organisms (GMOs) has become important for international trade of agricultural products. Concerns have been raised in the use of GM technology leading to potential health and environmental risks; one of these has been the proper labelling of food products produced using GM technology[2]. As the genetic modifications using recombinant DNA techniques may cause biosafety issues related to the environment and human health, methods that can detect transgenes in GM plants need to be put in place[3]. Polymerase chain reaction (PCR) has been widely used to confirm the presence of GMOs and to ensure the reliability of labelling systems[4–11].

High-yielding crop varieties with tolerance to abiotic stresses such as drought and salinity are always in demand for crop improvement programmes. Introduction of stress-tolerant genes from wild tomato species into tomato cultivars had initiated the breeding for resistance. Currently, modern molecular approaches are being utilized to develop cultivars that are tolerant to abiotic stresses[12–14]. Substantial progress has been made in the identification of genes involved in abiotic stress tolerance, and their transfer to crops of economic importance for increased stress tolerance. Osmotin is a stress-responsive protein adapted to salinity and desiccation, and accumulates in salt-adapted cells[15]. Overexpression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco[16]. Singh *et al.*[17] hypothesized that the synthesis of osmotin protein could induce synthesis and accumulation of certain solutes or could be involved in metabolic or structural changes.

Ouyang *et al.*[18] transformed tomato (*Lycopersicon esculentum* cv. 'A53') with a tobacco *osmotin* gene and a bean *chitinase* gene using *Agrobacterium* to develop germplasm with improved *Fusarium* wilt resistance. Using PCR and Southern blot hybridization, stable integration of the transferred genes into the genome at various insertion sites was confirmed.

In India, successful integration of *osmotin* gene through *Agrobacterium*-mediated transformation for enhancing cold tolerance has been undertaken in tomato variety 'Pusa Ruby'. *In vitro* selection against selectable marker kanamycin and PCR analysis of regenerated tomato plantlets for promoter (*CaMV*), *npt-II* (*neomycin phosphotransferase II*) gene and *osmotin* gene has confirmed the gene integration[12].

In 2006, Bansal and his group successfully developed transgenic tomatoes overexpressing *osmotin* gene, which were evaluated for drought and salt stress in the phytotron facility of the Indian Agricultural Research Institute (IARI), New Delhi, and the selected tolerant lines were tested in contained field trials[14].

PCR-based methods are the most common DNA detection methods for identifying the presence of GMOs[8]. These methods are sensitive, and require small aliquots of planting material for GM analysis. PCR methods are not only used for the identification of GM products, but also for quantification purposes[19]. To make the screening procedure robust, along with the detection of target sequences, plant species-specific reference gene should also be detected as an internal control. *LAT 52* (*Late Anther Tomato*) gene is an anther-specific gene from tomato abundantly expressed during pollen maturation and is present in a single copy in the tomato genome[20]. *LAT 52* gene has been successfully used as a tomato endogenous reference gene in both the qualitative and quantitative detection of transgenic tomatoes, even for some processed foods derived from transgenic and non-transgenic tomatoes[21].

Multiplex PCR (MPCR)[22] is a variant of the conventional PCR, which includes two or more pairs of primers in a single reaction to amplify corresponding genes simultaneously with the same sensitivity and specificity as PCR. MPCR has been used to detect the transgenes in different GM crops like soybean[23,24], maize[8,25,26], canola[27], squash[28] and cotton[29]. The advantage of multiplex methods is that fewer reactions are required to test a sample for the presence of GMO-derived DNA and it is more reliable and cost-effective.

In the present study, MPCR-based DNA assay for simultaneous detection of multiple target sequences, viz. *osmotin*

gene, *CaMV* 35S promoter sequence and endogenous *LAT 52* gene in GM tomato has been developed. Internal control target (*LAT 52* gene) was included both to assess the efficiency of all reactions and to eliminate any false negatives.

The seeds of two lines of transgenic tomato (line 528 and line 564) with *osmotin* gene along with the non-transformed tomato seeds were procured from National Research Centre on Plant Biotechnology (NRCPB), New Delhi, and were grown in the National Containment Facility at National Bureau of Plant Genetic Resources (NBPGR), New Delhi.

Genomic DNA was extracted and purified from fresh leaves of 5–6-week-old seedlings of transgenic as well as non-transgenic tomato lines using modified CTAB extraction method[30]. To tackle the problem of phenolics, 0.2% poly vinyl pyrrolidone (MW 40,000) was used in the extraction buffer. The quality of DNA was evaluated from the 260/280 nm UV absorption ratios and by agarose gel electrophoresis. The isolated DNA was quantified using VersaFluor™ Fluorometer. DNA samples were diluted to a final concentration of 5 ng/µl.

Two pairs of primers for detection of *osmotin* gene were designed using the sequence (GenBank accession no. X95308) of 2033 bp *Nicotiana tabacum* (common tobacco) *osmotin* gene with Primer Select 5.05 software (DNASTAR Inc., USA; Table 1). For the detection of endogenous *LAT 52* gene, the primer pair Lat1/ Lat2 amplifying 92 bp band and for 35S promoter, the primer pair 35S-F/R amplifying 195 bp were used[21,31]. The primers were synthesized by Operon Technologies, Inc., USA and Bioserve Biotechnologies (India) Pvt Ltd, India.

The protocols for individual as well as for MPCR were standardized using different PCR components and temperature regimes for each primer pair to detect *osmotin* gene, 35S promoter sequence and endogenous *LAT 52* gene in the transgenic tomato lines. PCR was performed in 20 µl reaction mixture containing 25 ng template DNA, 1X polymerase buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 U *Taq* DNA polymerase and 0.2 µM each of forward and reverse primers. Gradient PCR was performed to select the annealing temperatures at which the primers give best results. Annealing temperatures ranging from 50 to 63°C and 52 to 60°C were used for osmotin 1 and osmotin 2 primer sets. Amplification reactions for *osmotin* gene using PCR was performed in PTC-200 Programmable Thermal Cycler (BioRad) under the following programme: initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 30 s, primer annealing at optimum annealing temperature 59°C for 1 min, primer extension at 72°C for 1 min (in total, 35 cycles of the above programme was performed), and final extension at 72°C for another 8 min. MPCR was also performed under similar PCR conditions and temperature profiles using three sets of primers, viz. osmotin 1/osmotin 2, 35S and Lat. The PCR amplified products were resolved on 2% agarose gels stained with ethidium bromide using 1× TBE as the running buffer on horizontal electrophoresis, visualized under UV light and photographed using Gel Documentation System (Alpha Innotech, USA).

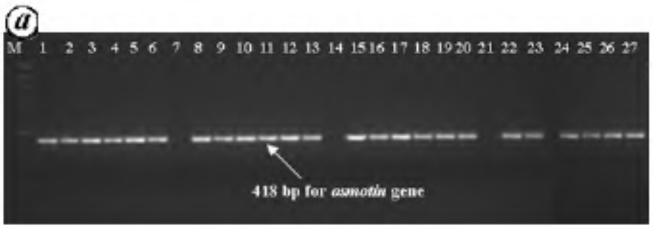
The two sets of primers for detection of the inserted *osmotin* gene were designed from the 2033 bp sequence (GenBank accession no. X95308) of *osmotin* gene in *N. tabacum* using Primer Select software. Gradient PCR (annealing temperatures ranging from 50 to 63°C and 52 to 60°C for osmotin 1 and osmotin 2 primer sets respectively) was performed to select the reliable annealing temperature. The most efficient primer annealing temperature for PCR reactions was 59°C for osmotin 1 and osmotin 2 primers. A 35-cycle protocol with 59°C annealing temperature using *osmotin* gene-specific primers was used. The results showed that the primers osmotin 1 and osmotin 2 amplified 418 and 353 bp products respectively, in the transgenic tomato lines 528 and 564, but no specific products were observed in the negative control sample (Figure 1 *a* and *b*). *LAT 52* gene was used as a tomato endogenous reference gene in the molecular detection of transgenic tomatoes and an amplicon of 92 bp was generated in both the transgenic lines as well as in non-transformed lines (Figure 2).

MPCR-based DNA assay for simultaneously detecting multiple target sequences in GM tomato was developed. MPCR was standardized to specifically amplify the *osmotin* gene, 35S promoter and *LAT 52* gene, an endogenous tomato gene in a single assay. A 35-cycle protocol with 59°C annealing temperature using *osmotin* gene, 35S promoter and endogenous *LAT 52* gene specific primers was used for simultaneous detection of these sequences in the transgenic tomato. In the first set of MPCR, the primers osmotin 1, 35S and Lat amplified specific products of 418, 195 and 92 bp respectively (Figure 3 *a*) in GM tomato. In another set, the primers osmotin 2, 35S and Lat amplified specific products of 353, 195 and 92 bp respectively, in GM tomato (Figure 3 *b*). No amplification products for *osmotin* gene and 35S promoter were detected in non-GM tomato, whereas 92 bp for endogenous *LAT 52* gene was amplified in non-GM tomato line.

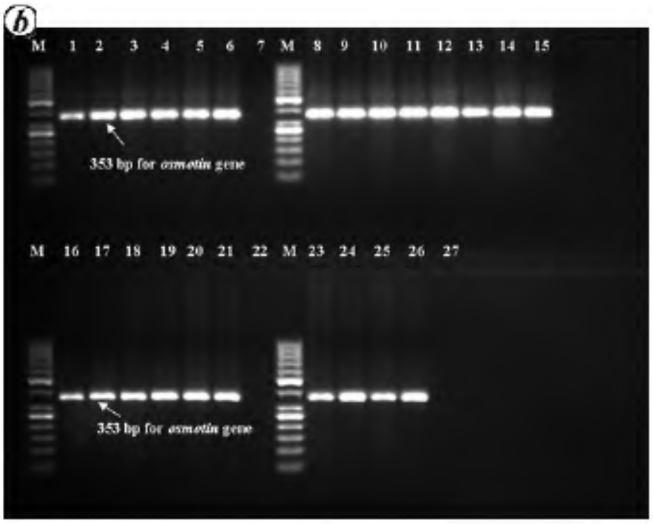
The GM crops have been approved for commercialization in many countries and some are in the pipeline of commercialization that have been approved for limited-scale field trials or multilocational field trials in India[1]. To meet the legislative and regulatory requirements, the development of reliable and sensitive assays for GM detection is necessary. PCR is the most widely used analytical method for both qualitative and quantitative analyses of GM crops. Several research groups[8,24–30] have developed a number of multiplex assays for GM detection, such as multiplexing for detection of five GM-maize events[32], viz. Bt11, Bt176, Mon810, T25 and GA21; detection of multiple target sequences in GM soybean (Roundup Ready™)[24]; detection of transgenes in GM

Table 1. Primers used for PCR amplification

Primer	Sequence (5′–3′)	Gene	Amplicon size (bp)
Osmotin-1-F	ACTTATGCTTCCGGCGTATTT	*Osmotin*	418
Osmotin-1-R	AACCAGGGCATTCACCATTTA		
Osmotin-2-F	CCGGTGATTGTGGTGGAG	*Osmotin*	353
Osmotin-2-R	ACTATAGGCGTCAGGACATCTTTG		
35S-F	GCTCCTACAAATGCCATCA	*CaMV* 35S	195
35S-R	GATAGTGGGATTGTGCGTCA	promoter	
Lat 1	AGACCACGAGAACGATATTTGC	Endogenous	92
Lat 2	TTCTTGCCTTTTCATATCCAGACA	*LAT 52* gene	



Lane M, 100 bp ladder; lanes 1, 3, 5, 8, 10, 12, 15, 17, 19, 22, 24, 26, Transgenic tomato line 528; lanes 2, 4, 6, 9, 11, 13, 16, 18, 23, 25, 27, Transgenic tomato line 564; and lanes 7, 14, 21; Non-transgenic tomato



Lane M, 100 bp ladder; lanes 1, 3, 5, 8, 10, 12, 14, 16, 18, 20, 23, 25; Transgenic tomato line 528; lanes 2, 4, 6, 9, 11, 13, 15, 17, 19, 21, 24, 26, Transgenic tomato line 564; and lanes 7, 22, 27; Non-transgenic tomato

Figure 1. Gradient PCR for detection of *osmotin* gene with primers (***a***) osmotin 1 amplifying 418 bp fragment and (***b***) osmotin 2 amplifying 353 bp fragment in transgenic tomato.

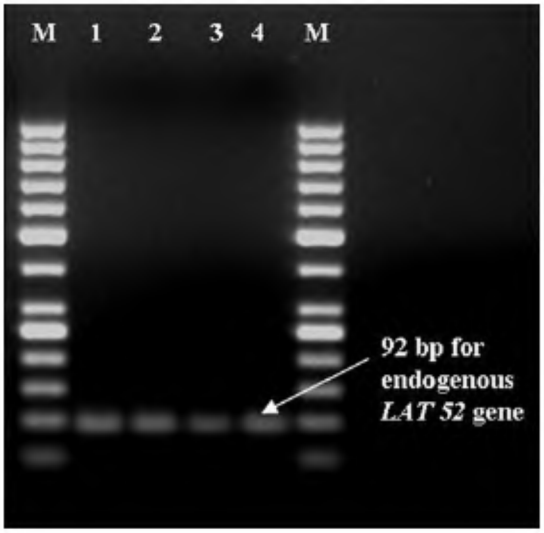
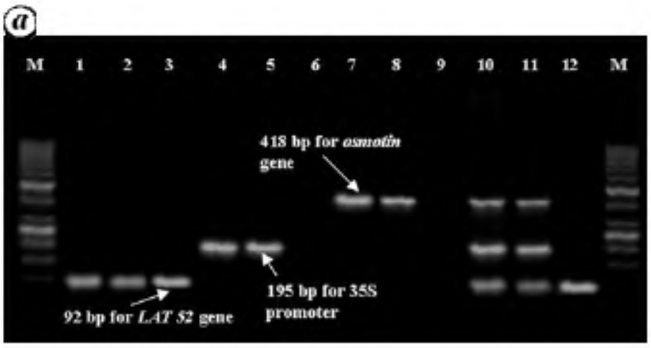


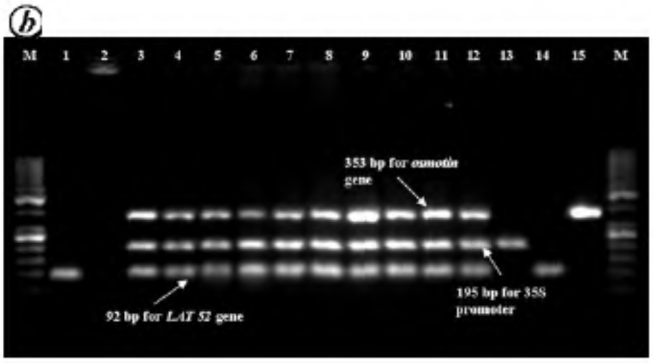
Figure 2. PCR for detection of endogenous *LAT 52* gene with Lat1/Lat2 primer amplifying 92 bp product in transgenic tomato. Lane M, 50 bp ladder; lane 1, Transgenic tomato line 528; lane 2, Transgenic tomato line 564, and lanes 3, 4, Non-transformed tomato lines.

tobacco with MPCR using seven primer pairs[3] and detection of 35S promoter and nopaline synthase (*nos*) terminator and anti-sense ethylene-forming enzyme (*EFE*) gene in GM tomato using MPCR and construct-specific PCR[33].

In India, salinity-cum-drought-tolerant tomato with *osmotin* gene is among the several transgenic events that have been approved for limited-scale field trials by the Review Committee on Genetic Manipulation, Government of India. In view of this, the present study was undertaken to develop MPCR-based protocol for simultaneous detection of *osmotin* gene, 35S promoter gene and endogenous *LAT 52* gene in GM tomato. Gradient PCR assays using two pairs of designed primers, osmotin 1 and osmotin 2, were performed to ascertain the optimum annealing temperature. The desired region of *osmotin* gene was amplified using the selected temperature range; however, 59°C was used as the optimum annealing temperature for better amplification in MPCR assays. For the detection of specific transgene *osmotin*, both the primers amplified the products of expected sizes and were further used along with primer pairs 35S-F, R[32] and Lat 1, Lat 2 (ref. 21) in MPCR in triplex reactions. A 35-cycle protocol with 59°C annealing temperature was used for simultaneous detection of three sequences in the transgenic tomato. The primer sets for *osmotin* were found equally effective in MPCR assays as that in individual PCR. The primer for tomato species-specific endogenous *LAT 52*



M, 100 bp ladder; lanes 1–3, *LAT 52* gene-specific primer (1-transgenic line 528, 2-transgenic line 564, 3-non-transgenic tomato); lanes 4–6, 35 S promoter specific primer (4-transgenic line 528, 5-transgenic line 564, 6-non-transgenic tomato); lanes 7–9, *Osmotin* gene-specific primer (7-transgenic line 528, 8-transgenic line 564, 9-non-transgenic tomato), and lanes 10–12, Multiplex using three sets of primers (10-transgenic line 528, 11-transgenic line 564, 12-non-transgenic tomato)



M, 50 bp ladder; lane 1, Non-transgenic tomato; lane 2, Water control; lanes 3–7, Transgenic line 528; lanes 8–12, Transgenic line 564; lane 13, Amplification with 35S primer only; lane 14, Amplification with Lat 1, Lat 2 only, and lane 15, Amplification with osmotin 2 only

Figure 3. Multiplex PCR for detection of *osmotin* gene, endogenous *LAT 52* gene and 35S promoter in transgenic tomato using primers (***a***) osmotin 1 and (***b***) Osmotin 2 along with 35S-F, R and Lat 1, Lat 2 primers.

gene was incorporated in the MPCR assay to evaluate the PCR efficacy, thus reducing the risk of false negatives. Amplification of the desired products of 418 and 353 bp for *osmotin* gene and 195 bp for 35S promoter were detected only in GM tomato lines, whereas *LAT 52* gene was amplified in both GM as well as non-GM lines as it is the endogenous reference gene for tomato.

The method reported in this study can considerably reduce the time and cost of detection of GM tomato with *osmotin* gene, especially for the screening of a large number of samples.

In the present study, to detect the *osmotin* gene, 35S promoter sequence and endogenous *LAT 52* gene in transgenic tomato, a robust MPCR protocol has been developed, which is highly sensitive and efficient. More than one target sequence can be detected in a single assay under the same reaction conditions. The reported detection method will be of immense use to meet the regulatory obligations and legal requirements.

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Production of andrographolide from adventitious root cultures of *Andrographis paniculata*

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Adventitious roots were induced directly from leaf segments of *Andrographis paniculata* on Murashige and Skoog (MS) medium with 5.3 µM α-naphthaleneacetic acid (NAA) and 30 g/l sucrose. Adventitious roots cultured in flasks using MS liquid medium with 2.7 µM NAA and 30 g/l sucrose showed higher accumulation of biomass (fresh and dry weight) and andrographolide within four weeks. Seven-fold increment of fresh biomass was evident in suspension cultures along with 3.5-fold higher andrographolide compared to natural plants. These results showed a great potentiality of adventitious root cultures for the production of andrographolide.

Keywords: Adventitious roots, *Andrographis paniculata*, Andrographolide, suspension cultures.

ANDROGRAPHIS PANICULATA Nees (Acanthaceae), commonly known as 'Kalmegh', has been widely used in India, Thailand, China and Malaysia for the treatment of hepatitis[1,2]. The plant is reported to possess protective activity against various liver disorders. The primary medicinal constituents of *A. paniculata* are andrographolide and related compounds which are diterpenoids showing antipyretic, antimalarial, anti-inflammatory, immunostimulatory and anticancerous activities[3–5].

Plant cell and organ cultures are promising technologies to obtain plant-specific valuable metabolites[6]. Cell and organ cultures have a higher rate of metabolism than field grown plants because the initiation of cell and organ growth in culture leads to fast proliferation of cells/organs and to a condensed biosynthetic cycle[7]. Further, plant cell/organ cultures are not limited by environmental, ecological and climatic conditions and cells/organs can thus proliferate at higher growth rates than the whole plant in cultivation[8]. Callus cultures of *A. paniculata* have been reported[9]. However, no andrographolides were detected in the cultures. In the present study, induction and culture of adventitious roots of *A. paniculata* were conducted and production of andrographolide in adventitious root cultures was investigated.

Young leaves were collected from field-grown plants of *A. paniculata* Nees and were washed thoroughly in

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