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Detection of nematicidal compounds and development of an *in vitro* mass multiplication protocol in Pusa Centenary *Chrysanthemum*

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ABSTRACT

Phytopathogenic nematodes are the major threat to agricultural crop productivity. Biopesticides can be safe and effective alternative to the harmful chemical pesticides. In this study the effect of *Chrysanthemum* cv. Pusa Centenary against *Meloidogyne incognita* was examined and the active chemical constituents were explored using LC-MS. To meet the growing demand protocol for *in vitro* mass propagation of Pusa centenary was also established. The aqueous extract of shoot and root showed significant mortality against second stage juveniles (J2s) and was directly related to the concentration and exposure time and it reached a maximum of 99.2% with 10% shoot extract after 72h. Insight into the chemical composition of Pusa Centenary using LC-MS revealed the presence of total 12 bioactive compounds (11 flavonoids and a terpene). The regeneration was obtained via somatic embryogenesis from petal explants in *Chrysanthemum* cv. Pusa centenary. Petal explants produced direct somatic embryos within 10-14 days on Murashige and Skoog (MS) media containing, 2, 4-Dichlorophenoxyacetic acid (2, 4-D) (1 mg L^{-1}) and ά-naphthalene acetic acid (NAA) (1 mg L^{-1}) along with 0.5 mg $L^{-1}$of 6-benzylaminopurine (BAP). On transfer to light on MS media with BAP (0.5 mg L^{-1}) and reduced auxins, the calli started turning green and proliferating into plantlets. The *in vitro* propagation protocol shall help in the mass production of this cultivar for isolation of these nematicidal compounds which may be used as a biopesticide (bionematicide).

Key words: *Chrysanthemum morifolium*, LC-MS, tissue culture, direct embryogenesis.

INTRODUCTION

Phytoparasitic nematodes (PPN) are recognized to be one of the greatest threats to the major food crops cultivated worldwide. The projected annual crop loss due to nematode infection is estimated to be $173 billion dollars worldwide (Singh et al., 16). *Meloidogyne incognita* are considered the most important obligate biotropic plant parasites for the reason that they are extremely widespread and accounts for 78 billion dollars crop loss globally (Kaur et al., 6). The use of chemical nematicides to control the nematodes are extensively employed since 1960s but the effect is only moderate. Also, the use of such chemicals is rather declining due to high production cost, low efficiency and poor specificity. Besides, nematicides application involves several risks: these compounds are sometimes phytotoxic and reflect great hazards to environment and human health by harming other soil micro biome. Henceforth, it is crucial to develop better and eco-friendly substitutes to control pathogenic nematodes for sustainable agriculture. Botanical pesticides can be one such effective alternative where natural compounds derived from plants exhibits antagonistic property against the target pest and are compatible with integrated nematode management (Oka *et al*., 13). Aqueous or alcoholic plant extracts of as many as 39 species have been demonstrated to possess nematicidal activity against *M. incognita*. Garlic (*Allium sativum*), *Eucalyptus chamadulonsis* (*camaldulensis*), and marigold (*Tagetes* spp.) exhibited nematicidal activity against *M. incognita* juveniles under the greenhouse conditions (Kamal *et al*., 5). Many reports demonstrated the nematicidal activity of several Asteraceae plants against root knot nematodes owing to their specialized biocidal metabolite content (Oka *et al*., 13). Leaf, root and flower extracts of *Calendula*, *Artemisia* and *Chrysanthemum* species have demonstrated to control plant parasitic nematodes due to their ability to produce nematicidal compounds against the juveniles and eggs of *M. incognita* (Bar-Eyal *et al*.,1). *Chrysanthemum* is the most attractive and popular flower cultivated for its diversified range of colors, shapes and shades. It is an herbaceous perennial of Asteraceae (Compositae) family and was first cultivated in china. They dominate the floriculture industry and draws in lucrative market prices. Pusa Centenary is a newly released variety of *Chrysanthemum* which is a gamma ray induced mutant of Thai Chen Queen. This cultivar is in demand due to its vigorous growing nature and it

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produces yellow flowers which remain fresh for 20 - 22 days in the field as well as in vase.

In our quest for the plant based nematicidal agents, the effect of aqueous shoot and root extract of *Chrysanthemum* cv. Pusa centenary against root knot nematode *M. incognita* was examined using *in vitro* assay and the active chemical constituents of the Pusa centenary were investigated using Liquid Chromatography-Mass Spectroscopy (LCMS). Moreover, to meet the demand in the market as well as to exploit this cultivar as a biopesticide, development of *in vitro* protocols for mass multiplication was inevitable. Hence, the protocol for *in vitro* mass propagation was also established.

MATERIALS AND METHODS

For the *in vitro* bioassay the aqueous shoot and root extracts were prepared as follows. The plant materials (1 g) were finely chopped and powdered prior to extraction. The ground material (1 g) was extracted thrice with sterile distilled water (SDW) using an FS30 Ultrasonic sonicator (Fisher Scientific, USA) at 40 kHz and 100 W for 30 min. at room temperature. The solvent was evaporated under reduced pressure below 35°C in a rotary evaporator (Heidolph, Germany) to obtain concentrate. The aqueous concentrate was stored at -20°C for further analysis. Adequate numbers of second stage juveniles (J2s) of *M. incognita* were obtained from pure stock culture maintained on roots of eggplant (*Solanum melongena* cv. Pusa Purple Long) in a glasshouse at ICAR- Indian Agricultural Research Institute, New Delhi, India as described previously (Whitehead and Hemming, 20). The bioassay was performed at room temperature, in 24-well polystyrene culture plates containing 500 µl per well, of three concentrations (2.5, 5.0, 7.5 and 10.0%) of aqueous extract of the root and shoot separately, SDW as control. Approximately 40 *M. incognita* J2s were introduced into each well. Each treatment was replicated in three wells. The mortality of the nematodes was determined using a stereo zoom microscope with 10 fold magnification after 24 h, 42h and 72h of the extract exposure. Juveniles were defined as dead if their body was straight and no movement was observed even after mechanical stimulation with needle (Fig. 1A). Percentage of J2 mortality was calculated for each well.

The LCMS analysis was performed with 1g of homogenized plant material using pestle motor. The ground material (1 g) was extracted thrice with methanol: water (25.00 mL, 80:20, v/v) using an FS30 Ultrasonic sonicator (Fisher Scientific, USA) at 40 kHz and 100 W for 30 min. at room temperature. The solvent was evaporated under reduced pressure below 35°C in a rotary evaporator (Heidolph, Germany) to obtain concentrate. The aqueous concentrate was stored at -20°C for further analysis. For LC-MS analysis, the extract was dissolved in HPLC grade methanol and filtered through a 0.45 µm nylon filter before analysis. The analysis was performed on an Agilent 1100 LC, coupled to a Quadrupole-Time of Flight mass spectrometer (QToF-MS, Synapt G2 HDMS, Waters Corporation, Manchester, UK).

For the regeneration experiment the flower petals of *Chrysanthemum* cv. Pusa Centenary were washed thoroughly in running tap water for 2 hours followed by washing with tween 20. Surface sterilization was done with 0.1% mercuric chloride for 5 minutes followed by three washes with SDW. These petals were cut into 3-4 mm fine sections using a sterile scalpel and were placed on the MS (Murashige and Skoog, *12*) media supplemented with 2, 4-Dichlorophenoxyacetic acid (2, 4-D) (1mg L^{-1}), ά-naphthalene acetic acid (NAA) (1mg L^{-1}) along with 6-benzylaminopurine (BAP) at 0.5 mg L^{-1} for callus induction. Thirty explants were inoculated as three replications of ten each. These explants were further sub-cultured onto the MS basal media with 2, 4-D, (0.1mg L^{-1}) and NAA (0.1mg L^{-1}) along with BAP at 0.5mg $L^{-1}$and maintained in dark at 25 ± 2°C for callus induction for 1-2 months. The number of cultures producing callus was observed at the end of 45 day. Well-developed somatic embryos were transferred to MS media with BAP (0.5mg L^{-1}) for plantlet formation for 30 days. The cultures were transferred to culture room with light maintaining a photoperiod of 16 h per day with temperature of 25 ± 2°C. The well-developed plants were then taken up for primary hardening. The well rooted plantlets were taken out from the culture media and washed with water carefully to remove the traces of agar and they were transplanted in jam bottles containing autoclaved mixture of Coco peat, Perlite and Vermicompost (2:1:1) for primary hardening and incubated in the same culture room in light at 25 ± 2°C. The plantlets (10-12 cm height) after sufficient growth were transferred from the bottles to plastic pots containing sterile mixture of sand, soil and vermicompost (1:2:1) and maintained in the polyhouse where in a temperature of 28±2°C with relative humidity of 65-75% was maintained for secondary hardening. The plantlets were irrigated with tap water twice in a week for a total period of two weeks to complete the acclimatization process.

RESULTS AND DISCUSSION

Plants have various natural products important for broad range antimicrobial activities against bacteria, fungi, virus and nematodes. In the present study, the nematicidal potential of *Chrysanthemum*

cv. Pusa Centenary was evaluated using *in vitro* assay. The most common test to evaluate the effect of Pusa Centenary on nematode mobility is the direct exposure of *M. incognita* juveniles to an aqueous plant extract. This method requires lesser quantity of the test material than plant bioassays and the results are obtained within a short span of time. The effect of aqueous shoot and root extracts of Pusa Centenary at different concentrations on mortality of *M. incognita* juveniles was observed overtime. Results showed that the shoot and root extracts showed remarkable nematicidal effects on *M. incognita* J2s at all concentrations throughout the experiment. Percentage of juvenile's mortality varied, depending on the concentration level and the exposure time (Fig. 1C-E). Shoot extract at 10% concentration resulted in 74.2%, 96.7% and 99.2% juvenile's mortality after 24h, 48h and 72h exposure time respectively, while the root extract showed 62.5 %, 71.7% and 73.3% death after 24h, 48h and 72h exposure time respectively The juvenile mortality increased significantly with plant extract concentration. Shoot extract was found to be more effective in causing highest juvenile mortality (99.2%) at 10% concentration after 72h, while the root extract showed less nematode mortality (73.3%) even after 72h exposure time at 10% concentration. There is significant increase in mortality with increase of the concentration while it achieved the highest mortality increase with 10% concentration and after the maximum exposure time. The study revealed that the Pusa Centenary plant extract are highly

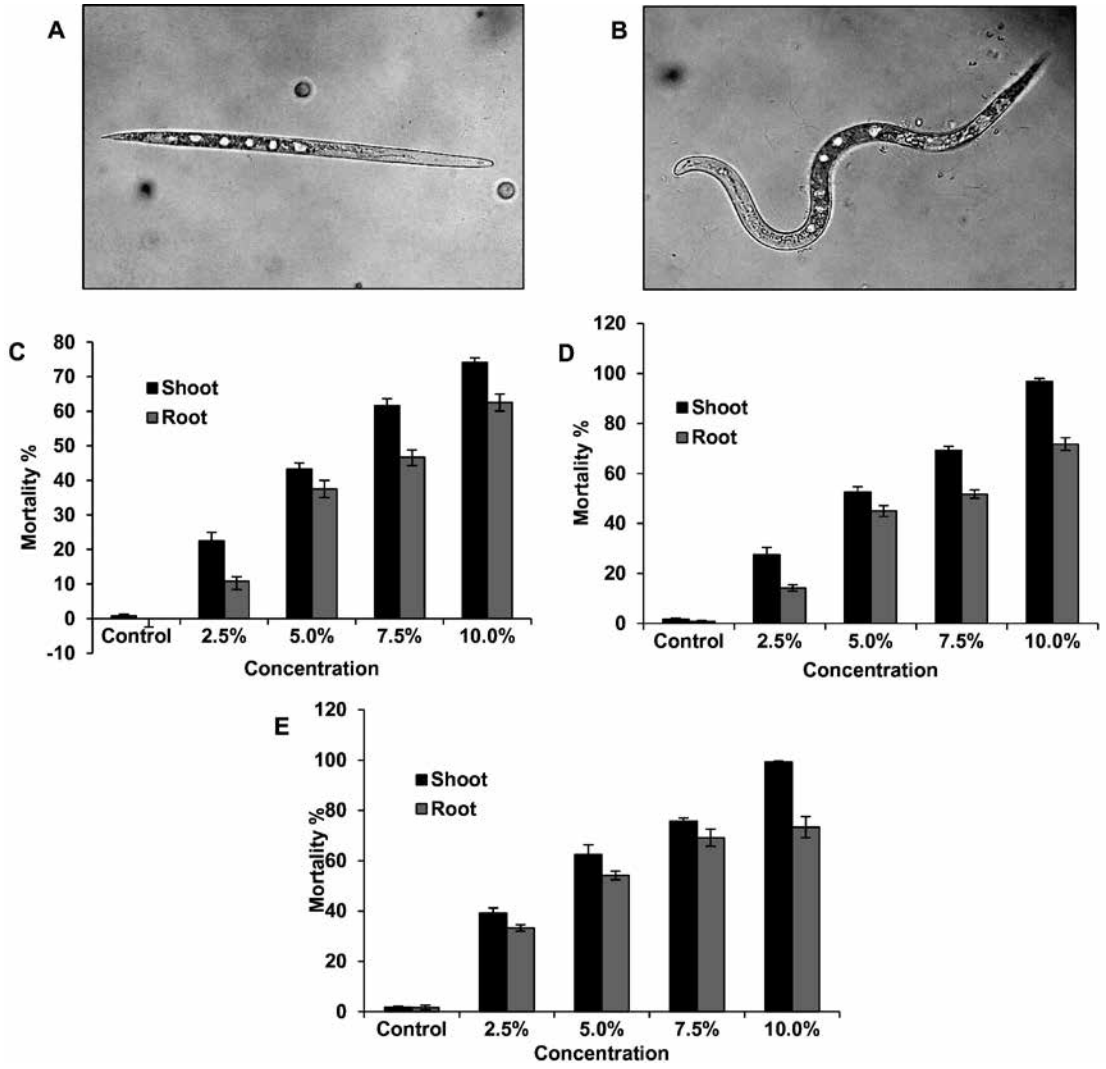

Fig. 1. Effect of aqueous root and shoot extracts of *Chrysanthemum* cv. Pusa Centenary on mortality % of second-stage juveniles (J2) of *Meloidogyne incognita*. (A) Dead *M. incognita* juvenile (B) Live *M. incognita* juvenile. Percentage mortality after (C) 24hr (D) 48h (E) 72h exposure time.

lethal to *Meloidogyne incognita* juveniles. To the best of knowledge this is the first report on the nematicidal activity of aqueous extract of a recently released variety *Chrysanthemum* cv. Pusa Centenary. Nevertheless, the nematicidal activities of the aqueous extract of *Chrysanthemum coronarium*, was reported against *M. incognita*, *M. javanica* and *M. artiellia* (Bar-Eyal *et al*., 1). Wild *Chrysanthemum* (*Chrysanthemum coronarium*) incorporated into the soil as green manure showed nematicidal activity against *M. incognita and M. javanica*, and nematode juveniles were killed by exposure to aqueous plant extracts *in vitro* (Bar-Eyal et al., 1). Plant parasitic nematode sensitivity towards nematicides varies among species and *Meloidogyne* J2s are highly sensitive to plant derived nematicides. The nematicidal plant extract are applied either as an aqueous extract or amended with organic materials as mulch.

Diverse range of organic compounds present in a single plant tissue acts synergistically to confer nematicidal properties, leading to high nematode mortality. The presence of several phenol groups act as chemical barrier against various pathogens thereby protecting the plant. Earlier reports revealed the antibacterial, antifungal, antiviral and anti-inflammatory activities in flowers and leaf of compositae plants (Matsuda et al., 10), still some of the bioactive components are unidentified. To identify the naturally occurring nematicidal compounds, LCMS of *Chrysanthemum* extract has been carried out. The LC-MS chromatogram of *Chrysanthemum* cv. Pusa Centenary extract is shown (Fig. 2). The structures of some of the phenolic compounds identified in the aqueous extract of *Chrysanthemum* are shown in Fig. 3. The retention times (tR) in LC, molecular ions [M+H]+, and major fragment ions of the detected peaks are listed in Table 1. Positive identification of the compounds in (Table 1) was made based on the comparison of retention times, and literature reports mass spectra for either authentic standards and/or their fragmentation pattern. Further the calculated molecular ions of all the compounds were matched with the actual molecular wt. of the standards including acceptable δ error mass value below 3 ppm level. Flavonoids are polyphenols that constitute a large class of secondary metabolites present widely in all plants. Flavonoids originate from shikimate and acetate pathway and contain a diphenylpropane backbone (C3-C6-C3). Flavonoids role is not only restricted to plant development but is also associated with attributing enhanced resistance to pathogens including nematodes (Chin et al., 2). Thus, total of 11 flavonoid compounds and one diterpene phytol of this extract were positively identified as 6-C-xylosyl-8C-glucosylapigenin, acacetin-7-O-rutinoside, acacetin-7-O-6”-malonylgalactoside,

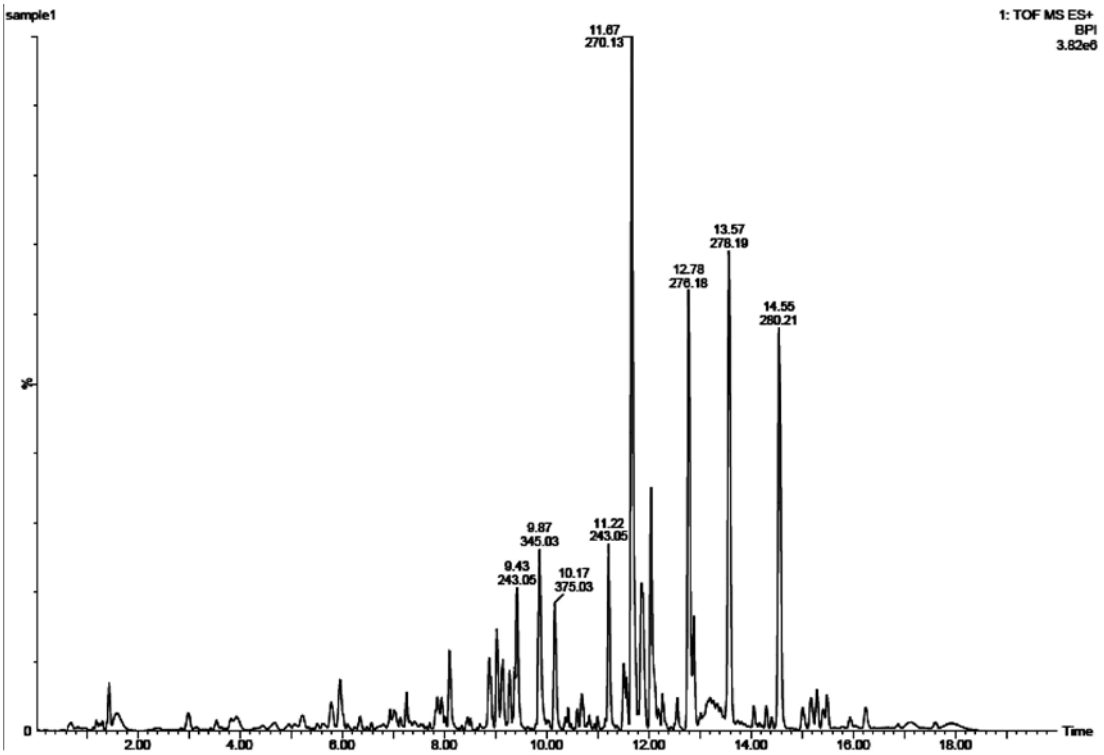

Fig. 2. LC-MS Total Ion Chromatogram (TIC) of *Chrysanthemum* cv. Pusa centenary aqueous extract.

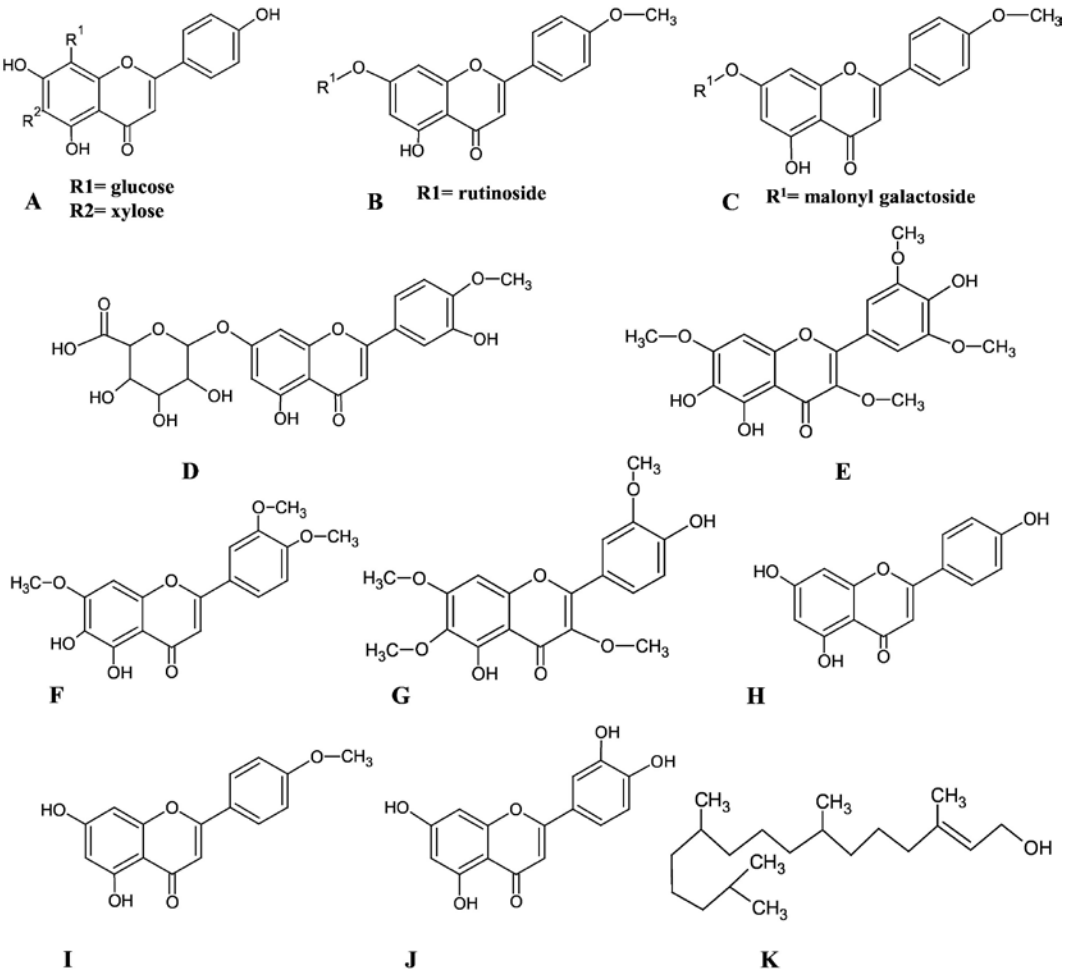

Fig. 3. Identified compounds in aqueous extract of *Chrysanthemum* cv. Pusa centenary. 6-C-xylosyl-8C-glucosylapigenin, acacetin-7-O-rutinoside, acacetin-7-O-6”-malonylgalactoside, diosmetin-7-O-glucuronide, chrysosphenol C, eupatorin, chrysosplentin, apigenin, acacetin, luteolin and phytol.

Table 1. Peak assignments of the aqueous extract of *Chrysanthemum* cv. Pusa Centenary.

Sl. No.	t_R (min.)	$[M+H]^+$ (m/z)	Aglycone/diagnostic fragments (m/z)	Identification
1	3.94	566.0380	163, 434, 547, 565	6-C-Xylosyl-8C-glucosylapigenin
2	8.01	594.0717	217, 285, 447, 549, 593	Acacetin-7-O-rutinoside
3	8.90	534.0250	165, 203, 285, 399, 533	Acacetin-7-O-6”-malonylgalactoside
4	9.15	533.0225	287	Luteolin-7-O-6”-malonylglucoside
5	9.28	477.0198, 499.0198 $(M+Na)^+$	301	Diosmetin-7-O-glucuronide
6	9.43	362.0207	361	Chrysosphenol C
7	9.87	362.0207	274, 344	Eupatorin/chrysosphenol
8	10.17	375.0309	344, 359, 374	Chrysosplentin
9	11.67	271.1322	-	Apigenin
10	12.01	284.1417	274, 285	Acacetin
11	12.05	287.1572	-	Luteolin
12	13.57	279.1945	249, 263, 278	Phytol

luteolin-7-O-6"-malonylglucoside, diosmetin-7-O-glucuronide, chrysosphenol C, eupatorin, chrysosplentin, apigenin, acacetin and luteolin. Apigenin and its glycoside, xylosyl-glucosyl-apigenin was identified from retention time (R_t) 11.67 and 3.94 min interval in LC. Sharp molecular ion peaks $[M+H]^+$ at m/z 271.1322 and 566.0380 was observed in the total ion chromatogram corresponding to apigenin and xylosyl-glucosyl-apigenin, respectively. Apigenin is a flavonoid abundantly present in many plants. Similarly, acacetin, acacetin-7-O-rutinoside andacacetin-7-O-6"-malonyl-galactoside was also identified from the spectra at the R_t value of 12.01, 8.01 and 8.90 min, corresponding to their characteristic molecular ion$[M+H]^+$ peaks at m/z 284.1417, 594.0717 and534.0250, respectively. Luteolin and its glucoside, luteolin-7-O-6"-malonylglucoside were also characterized from their molecular ion peaks $[M+H]^+$ at m/z 287.1572 and 533.0225 with the δ error mass value below 3 ppm level. Both of these compounds were eluted from the column 12.05 and 9.15 min. interval. Mass fragmentation pattern of luteolin-7-O-6"-malonyl-glucoside showed sharp daughter ion at m/z 287 which was originated due to loss of malonyl-glucoside moiety (247 amu). Apigenin and its derivatives including acacetin and luteolin revealed nematicidal activity by inducing the larval growth inhibition in *Caenorhabditis elegans* (Kawasaki et al., 7) reported high nematicidal activity of many phenolic compounds including acacetin and apigenin. Some of the identified compounds such as luteolin and its glycosylated derivative have already been reported in *C. coronarium* and other *Chrysanthemum* (Clifford et al., 3). These results agree with those that reported that *Chrysanthemum* species produces a wide variety of bioactive compounds such as flavonoids, phenols, phenolic acids and terpenes (Matsuda et al., 10; Hu et al., 4). Many of these compounds especially the flavonoids have demonstrated anti-inflammatory and anti-cancer properties. For instance, acacetin 7-O-galactoside and apigenin 7-O-beta-D- (4"-caffeoyl) glucuronide isolated from *C. morifolium* exhibited anti-human immunodeficiency virus type 1 (HIV-1) action (Hu et al., 4). Other flavonoids like luteolin 7-O-glucoside and luteolin-7-O-glucuronide are known to possess a variety of biological properties, such as antioxidant, anti-virus, anti-HIV, anti-inflammatory, anti-carcinogenic, anti-mutagenic and anti-hepatotoxic activities that are considered advantageous to human health (Parejo et al., 14; Miyazawa and Hisama., 11). Luteolin present in the flower of *C. morifolium* possesses strong anti-cancer property together with other chemotherapeutic agents (Shi et al., 15). Flavonoids such as luteolin and luteolin 7-O-(6"-O-malonyl)-glucoside obtained from the petals of *Chrysanthemum* had shown to suppress CCl-4 induced liver injury in mice (Sugawara and Igarashi., 18). Apigenin and its derivative apigenin 7-O-glucoside have been identified in flower of edible *Chrysanthemum* (*Chrysanthemum×morifolium* Hemsl). Previously luteolin, luteolin 7-O-glucoside, apigenin 7-O-glucoside, acacetin 7-O-glucoside, luteolin 7-O-(6"-O-acetyl)- glucoside and acacetin 7-O-(6"-O-acetyl)-glucoside were identified in *Chrysanthemum×morifolium* Ramat (Wang et al., 19).

To meet the growing demand of this cultivar and to make available large number of plants for extraction of nematicidal compounds *in vitro* micropropagation of *Chrysanthemum* Pusa centenary was also attempted. In our *in vitro* micropropagation experiment, 10 petal explants were inoculated as three replications in MS media supplemented with 2, 4-D, (1 mg L^{-1}) + NAA (1 mg L^{-1}) +BAP (0.5 mg L^{-1}) and there was 100% response on this media for direct embryogenesis. There was profuse direct embryo formation from the surface within two weeks of culture (Fig. 4). At the end of one month when they were observed under the stereo microscope the clear globular embryos with suspensor region was seen (Fig. 4). An average of 54 distinct somatic embryos was observed from a single explant. These bipolar somatic embryos with root and shoot pole could be observed on subculture to fresh MS basal media with BAP (0.5 mg L^{-1}) in light, they regenerated into plantlets (Fig. 5). From a single petal explant we were able to obtain 18-20 plantlets. After sufficient root growth the plantlets were removed for hardening. The well rooted plantlets were taken out from the culture and washed with water carefully to remove the traces of agar and they were transplanted in jam bottles containing autoclaved mixture of Co-copit, Perlite

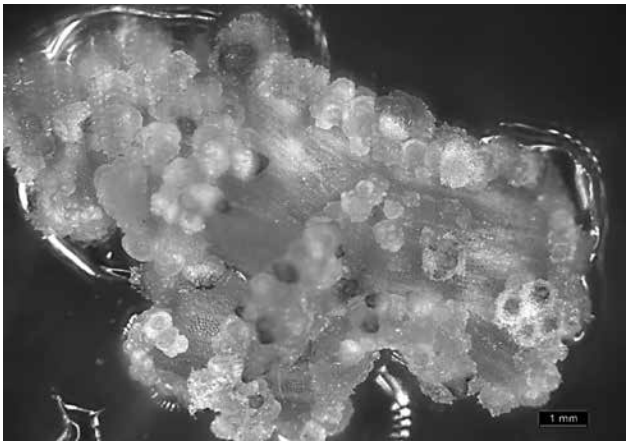

Fig. 4. Direct embryo formation from the surface of the petal explant within two weeks of culture on 2, 4-D, (1 mg L^{-1}) + NAA (1 mg L^{-1}) +BAP (0.5 mg L^{-1}).

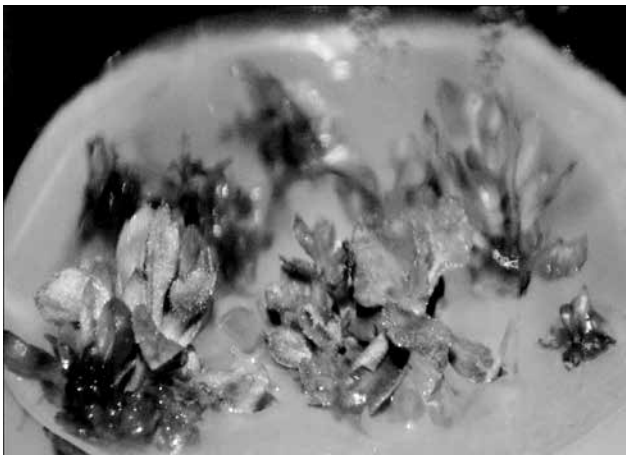

Fig. 5. Somatic embryos with root and shoot pole were separated singly and regenerated into plantlets on transfer to fresh MS basal media with BAP (0.5 mg L^{-1}) in light.

and Vermicompost (2:1:1) for primary hardening and incubated in the same culture conditions for a month (Fig. 6). After a month the well grown plantlets from the bottles were transferred to pots containing sterile mixture of sand, soil and vermicompost (1:2:1) and they were transferred to the polyhouse where in a temperature of 28±2°C with relative humidity of 65-75% was maintained for secondary hardening (Fig. 7). The plantlets were irrigated with tap water twice in a week for a total period of two weeks to complete the acclimatization process. There was 90-98% survival of plants. The response from petals is seen within 10-14 days and it is able to produce direct embryos and this may be attributed to the meristematic

Fig. 6. Primary hardening of *in vitro* regenerated plantlets in jam bottles containing autoclaved mixture of Co-copit, Perlite and Vermicompost (2:1:1) for one month.

Fig. 7. Secondary hardening of *Chrysanthemum* cv. Pusa centenary in polyhouse.

nature of the petals. This kind of quick response and meristematic nature of petals of *Chrysanthemum* have been reported previously (Song *et al*., 17). The effect of growth plant growth regulators on adventitious shoot regeneration from leaf, stem, petiole and petal explants of *Chrysanthemum* was studied and it was reported that petals are the most responsive for shoot regeneration (Song *et al*., 17). A high concentration of auxin IAA along with BAP and Kinetin was required for shoot regeneration from different explants. In *Chrysanthemum* a high genotypic influence has been reported by many researchers. In the earlier report on direct embryogenesis (Mandal and Datta, 9) only seven embryos per explant could be obtained but we have observed an average of 54 distinct somatic embryos from a single explant in our study. For example, in a study of somatic embryogenesis from 10 cultivars of the Lady group it is reported that the highest response for somatic embryogenesis was in Lady Salmon and in Lady yellow (Lema-Rumniska and Niedojadlo, 8) when the 2, 4-D was at a concentration of 4mgL^{-1}. Direct embryogenesis from ray florets of five cultivars of *Chrysanthemum* with a maximum response was 40% with the production of 7 embryos per explants was reported (Mandal and Datta, 9). In our current protocol the advantage is that we are able to obtain somatic embryogenesis with low concentrations of auxins and also obtained higher percentage embryogenesis and somatic embryos from the cultivar Pusa Centenary which proves its amenability for large scale culture.

In summary, the results of the present study evidently indicate that the extracts of Pusa Centenary

can be used to formulate a commercial plant based nematicide. Although plant materials and extracts can be used for sustainable organic farming systems, extraction and identification of the nematicidal compounds is necessary for further advancement of commercial products. A more promising means of developing a bionematicide will be the synthesis of compounds with higher nematicidal activity based on *Chrysanthemum* cv. Pusa Centenary derived or related compounds. The regeneration protocol established here can be used for multiplication of this cultivar in large number to isolate nematicidal compounds and also to meet its huge market demand.

DECLARATION

The authors declare no conflict of interest.

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