Research Article

Effect of Additives on Plant Regeneration in *Hedychium coronarium* J. Koenig an Endangered Aromatic and Medicinal Herb

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ABSTRACT

Fortification of culture media with different plant growth regulators i.e. auxins and cytokinins is not enough to regenerate the plant with high efficiency. Among all the additives used PG and AC acted positively for multiple shoot regeneration from *in vitro* regenerated shoots. Maximum shoot length (10.86±0.16) was obtained on lower concentration of PG (1.0 mgL^{-1}) and maximum shoot number (6.56±0.14) on the same concentration with high frequency of shoot initiation within 3 weeks of inoculation. Maximum root length (19.80±0.14) was obtained on NAA (1.0 mg L^{-1}) after 4 week of sub culturing and maximum root number (14.61±0.11) resulted, after 6 weeks on 1.0 mg L^{-1} NAA. The best rooting occurred on liquid half strength MS basal medium supplemented with NAA (1.0 mg L^{-1}). The plantlets developed were hardened and established in natural soil with 80 % survival.

Keywords: Activated charcoal, Additives, *Hedychium coronarium*, Phloroglucinol, NAA.

INTRODUCTION

Gulbakawali *(Hedychium coronarium* J. Koenig, family- Zingiberaceae) is an aromatic erect herb. It is valued for its anti cancerous, anti diabetic, antimalarial, anti-inflammatory, indigestion, properties possessed by its various parts. This plant possesses a terpenoids viz. coronarin A, B, C, D, E & F. Coronarin A is used to inhibit the proliferation of human umbilical vein, shown to be cytotoxic in tumor cells[1]. Coronarin D is known to inhibit the release of h-hexosaminidase from RBL-2H3 cells[2,3] and acetic acid induced vascular permeability in mice.[4] It is distributed up to about 1,900 m in tropical and sub tropical Asia, probably from the Himalayan foothills of Nepal east to China and South through Indo-China.[5] In India, it is distributed throughout the country and found mainly in moist tropical forest near stream and canals or near water channel. Owing to indiscriminate collection, over exploitation for commercial and domestic utilization, difficulty in propagating plant through seeds is yet another factor responsible for its infrequent occurrence, this valuable herb become endangered in Vindhyan region in Madhya Pradesh, central India and in its native ranges[6-9] and has been reported as red listed in Manipur[9].

Regeneration and reestablishment of plant through *in vitro* culture is one of the most effective biological techniques to conserve biological diversity. Optimum factors influencing growth and morphogenesis vary with the genotype and types of explants used for Micropropagation.[10] Murashige and Skoog (MS) medium with a high content of nitrate, ammonium and potassium is of widespread use in the successful culture of a wide variety of plants. Sometimes it requires supplementation of additional substances in the medium. Application of additives is adapted to the cultural needs.[11] In this paper the effect of some additives like activated charcoal (AC), casein hydrolysate (CH), coconut milk (CM), silver nitrate (AgNO3) and phloroglucinol (PG) their impact on the direct *in vitro* multiplication of *Hedychium coronarium* is described.

Activated charcoal (AC)

Activated charcoal is a fine powdered wood charcoal added to tissue culture media, to bring about changes in the composition of the medium.[12] Growth promoting effects of AC have been attributed to its capacity to adsorb unwanted phenolic exudates which accumulates in culture media, especially in the early stages of culture initiation.[13] AC has a very fine network of pores with large inner surface area on which many substances can be adsorbed.[14] In *in vitro* conditions, the activated charcoal is commonly used in tissue culture media. Its addition to culture medium many promote or inhibit growth *in vitro*, depending on different factors. The positive or negative effects of activated charcoal depend especially on its concentration in the culture medium, species cultivated in vitro and their phases of multiplication.[15, 16]

Casein hydrolysate (CH)

Casein hydrolysate (CH) is an organic nitrogen supplement containing a mixture of amino acids and is good source of reduced nitrogen in many tissue culture systems.[17] It can be a source of calcium, phosphate several microelement vitamin and most importantly, a mixture of up to 18 amino acids.[18] It has been thought that nitrogen deficiency can cheaply be fulfilled by its addition[19] presumably it contains some stimulatory factors yet unidentified.

Silver nitrate ($AgNO_3$)

Silver nitrate play a major role in influencing somatic embryogenesis, shoot formation and efficient root formation which are the prerequisites for successful

genetic transformation.[20] Addition of $AgNO_3$ to the culture media has been reported to greatly improve the regeneration of dicot and monocot plant tissue cultures.[21, 22] The precise molecular mechanism of $AgNO_3$ action on plants is unclear.

Coconut milk (CM)

It is an undefined supplement derived from the liquid endosperm of *Cocos nucifera* fruits whose composition can vary considerably.[23] Coconut milk has been shown to have cytokinin activity and hence can induce plant cells to divide and grow rapidly. It was first used in tissue cultures by[24] for the development of very young embryos of *Datura stramonium*. The liquid has been found to be beneficial for inducing growth of both callus and suspension cultures and for the induction of morphogenesis.

Phloroglucinol (PG)

Phloroglucinol is a phenolic compound predominately found in xylem sap of apple and is known to promote growth and development in a number of plant species.[25] Phloroglucinol added to culture media may enhance growth and rate of axillary shoot growth in shoot tip cultures of several woody plants.[26]

MATERIALS AND METHODS

Underground rhizome of *H. coronarium* was collected from Jawahar Lal Nehru Krishi Vishwa Vidhyalay (JNKVV) Jabalpur, (M.P). Plants were identified by institutional Botanist and voucher specimen (No. 1993) of healthy and vigorous plants were chosen for study. The characteristic bud/eye of the plant rhizome was used as explant. Rhizome was excised and washed thoroughly under running tap water for 30 min to wash off the adhering soil particles. Roots and outer scales were removed and the rhizome was washed with 0.01% Labolene (Qualigens, India) for 10 min, followed by repeated washings with tap water. The explants were then transferred to laminar air flow hood where they were surface sterilized in 70% ethanol for 5 min, washed with sterilized distilled water 2-3 times and subsequently treated with 0.1% $HgCl_2$ for 5 min. Explants were finally washed with sterile distilled water (3-4 times). Explants were cut into segments of 5-8 mm X 4-5 mm size, dried on sterile filter paper and aseptically transferred onto solidified MS medium. *In vitro* raised plants (0.5-1 cm) in basal medium served as the source of explants which were inoculated on MS medium supplemented with different concentrations (0.1, 0.5, 1.0 and 5.0 mg L^{-1}) of additives viz. Activated charcoal (AC), Silver nitrate ($AgNO_3$), Casein hydrolysate (CH), Phloroglucinol (PG) and Coconut milk (CM %) along with BAP (1.0 mg L^{-1}) with 3% (w/v) sucrose and 0.8% agar. The pH of the medium was adjusted to 5.6-5.8 (before adding agar) with 1 N NaOH and 1 N HCl prior to dispensing into culture tubes (15 X 150 mm). All the cultures were maintained at 25 ± 20^0 C and photoperiodic cycle of 16 hr light [approx 1500 lux] and 8 hr dark provided by Philips cool white fluorescent tubes. The cultures were transferred onto fresh culture medium at every 4 weeks interval. All experiments were conducted in triplicates containing 12 explants each for each treatment.

Rooting of *in vitro* induced shoots

After four months of culture, the multiple shoots were singled out from the parent tissue and transferred to the rooting medium containing full strength MS medium, 3% (w/v) sucrose, devoid of agar (liquid medium) supplemented with different concentrations of IAA, IBA and NAA (0.1, 0.5, 1.0, 5.0 mg L^{-1}). Data were recorded as percentage of rooting, mean number of roots per shoot and root length after four weeks of culture.

Acclimatization of Plantlets

Six months old plantlets (10-12 cm) of *H. coronarium* were taken out from the test tubes carefully and washed with tap water to remove traces of medium, then treated with bavastin (0.1% w/v) for 2 mins followed by 2-3 rinse with tap water. The plantlets were then transferred to thermocol cups containing autoclaved soil, sand and farm yard manure (1:1:1). To maintain the humidity, plants were completely covered with plastic bags, which were removed progressively to aid adoption to normal environmental conditions. Plantlets were covered with plastic bags. After 2 weeks, polythene bags were perforated and plants were irrigated with sterilized distilled water for gradual acclimatization. The plantlets were thus successfully hardened with 85 % survival rate and transferred to field.

Statistical analysis

All the experiments were repeated three times with 12 replicates. Data were recorded at 30 day interval. Data on multiple shoot production and rooting were statistically analysed and means were separated at $p= 0.05$ level of significance using DMRT using SPSS 20.

RESULTS

Activate charcoal

Activated charcoal has been reported to inhibit heavy leaching of phenols.[27] The presence of activated charcoal in the regeneration medium has a significant influence on the development of shoots *in vitro*.[28] In the present study activated charcoal show positive effect on plant, it increases shoot length (4.39±0.15) at lower concentration after 5 weeks of culture on AC (0.1 mg L^{-1}) and at same concentration it shows multiple shoot formation (3.75±0.45) (Plate 2, Figures 5a-5d). The shoots formed in the presence of activate charcoal are healthy, green in colour with long leaves. White roots are present at its base. AC favours efficient rooting from *in vitro* regenerated shoots. But at higher concentration the results obtained are not satisfactory; low shoot length and shoot number was observed (Table 1).

Casein hydrolysate

Casein hydrolysate might contain some unknown growth promoting factor.[29] In our study casein hydrolysate was

supplemented to the selected medium, supports shoot elongation and shoots multiplication. New shoot was obtained after 3 weeks of culture. Maximum shoot length (5.56±0.12) was obtained within 9 weeks on higher concentration of CH (5.0 mg L^{-1}) with maximum number of shoots (3.53±0.45) (Plate 1, Figures 2a-2d) (Table 1). Lower concentration of casein hydrolysate was unsuitable (Graphs 1-2).

Silver nitrate

The explants remained unresponsive when cultured on MS medium fortified with silver nitrate. All the concentrations show very low shoot multiplication and elongation. The shoots obtained were fragile and light green in colour (Plate 1, Figures 1a-1d) (Table 1) bearing small leaves (Graphs 1-2).

Coconut milk

The explants remained totally unresponsive when cultured on MS medium fortified with CM. Significant reduction in frequency of shoot number and shoot length was found. Shoots developed were yellowish in colour with very low shoot initiation. Small shoots with 1-2 leaves (Plate 1, Figures 3a-3d) (Table 1) was observed.

Phloroglucinol

Maximum frequency of shoot initiation has been observed on PG supplemented medium on *in vitro* raised plants among all the additives tried (Table 1, Graphs 1-2). Explants when treated with different concentration of PG with BAP (1.0 mg L^{-1}) resulted in the formation of healthy shoots bearing large dark green leaves in 6 weeks (Plate 2, Figs 4a-4d). Phloroglucinol has produced positive effect on all the shoot regeneration parameters FSI (Frequency of shoot initiation), MSN (Mean shoot number), MSL (Mean shoot length) (Table 1) tested for regeneration of *H. coronarium* by developing healthy plantlets (Plate 2). Maximum shoot length (10.86±0.16) was obtained on lower concentration of PG (1.0 mgL^{-1}) and maximum shoot number (6.56±0.14) on the same concentration with high frequency of shoot initiation within 3 weeks of inoculation.

Shoots with 6 to 8 cm length were excised and rooted on full strength MS medium. After 2 week of incubation, rooting was observed on MS medium. In order to increase root number and reduce the time for rooting, different concentrations of NAA, IAA and IBA was added. Maximum root length (19.80±0.14) was obtained on NAA (1.0 mg L^{-1}) (Graph 3) after 4 week of sub culturing and maximum root number (Figure 6a-b), (Graph 3) was (14.61±0.11) resulted, after 6 weeks on 1.0 mg L^{-1} NAA (Table 2).

Acclimatization of plantlets can be considered as one of the most important phase in tissue culture techniques. The well rooted plants after 8-10 weeks were transferred to plastic cups containing soil, sand and farmyard manure and kept under controlled condition (Figure 1 i, l). Upon transfer to the mixture the plants started to produce new shoots and roots after four weeks of hardening. Later they were transferred to field with 80 % survival rate.

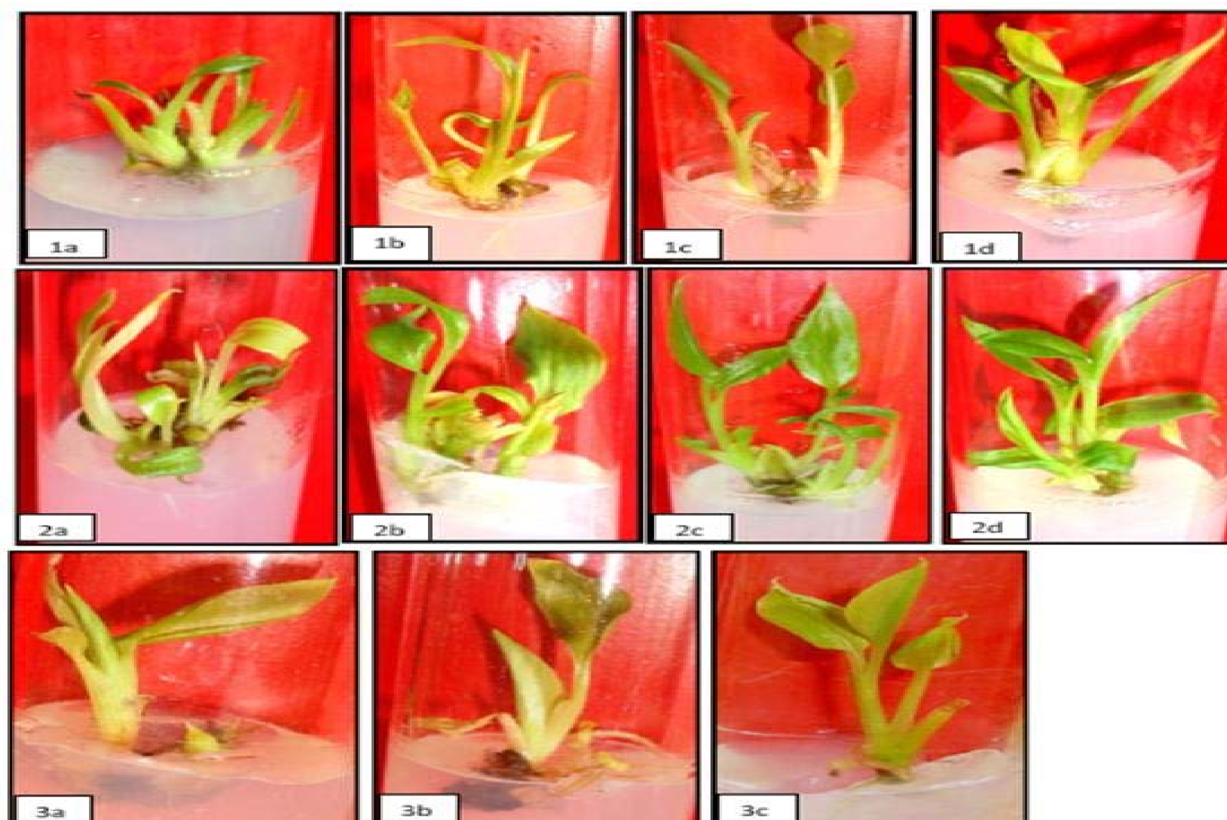


Plate 1: Effect of additives on multiple shoot formation and elongation in *Hedychium coronarium* J. Koenig from *in vitro* raised plants: Effect of $AgNO_3$ on shoot elongation (1a-1d), Effect of CH on shoot multiplication (2a-2d), Effect of CM on shoot elongation and multiplication (3a-3c).

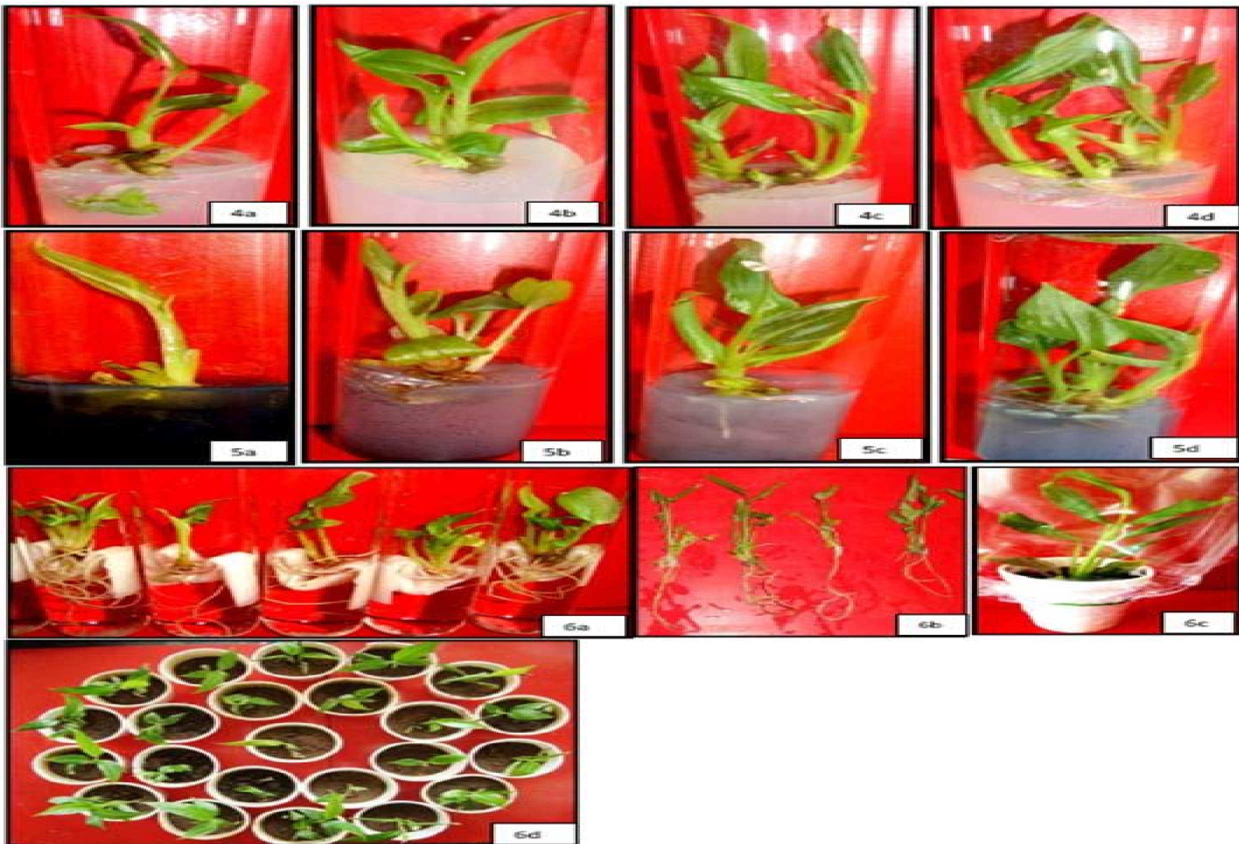
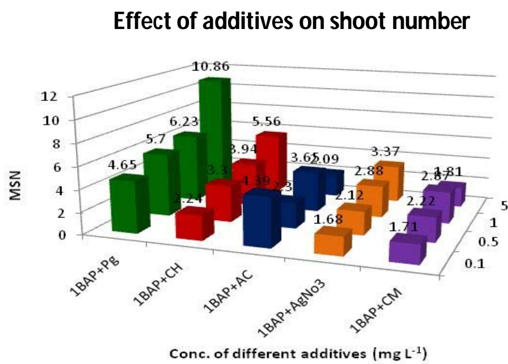
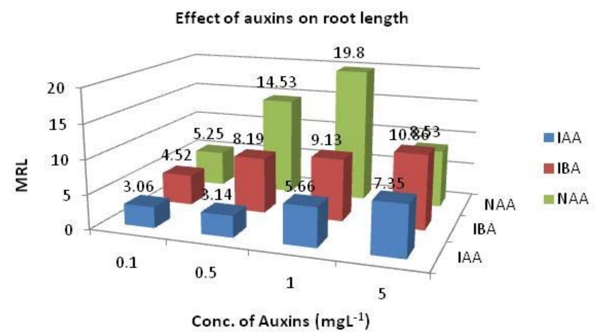


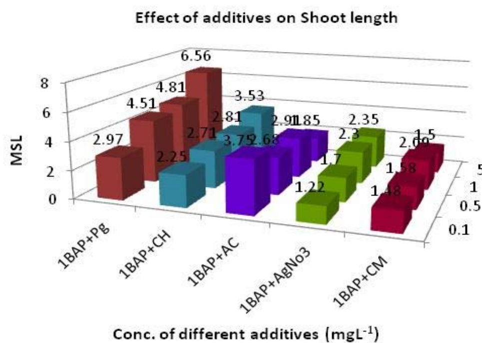
Plate 2: Effect of additives on multiple shoot formation & shoot elongation in *Hedychium coronarium*: Effect of PG on shooting & elongation (4a-4d), effect of activated charcoal on shoot elongation (5a-5d), i*n rooting* in *H. coronarium* (6a-6b), Regenerants showing hardening (6c) and acclimatization (6d).



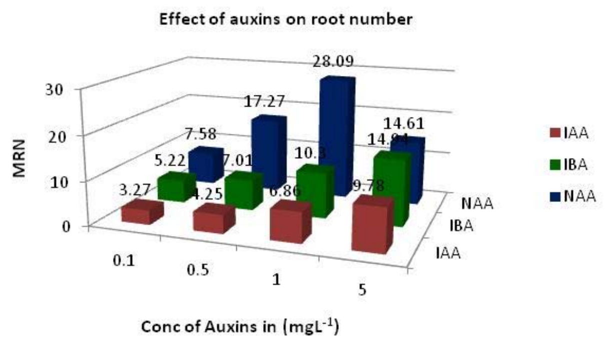
Graph 1: Effect of additives on Shoot multiplication on *Hedychium coronarium*



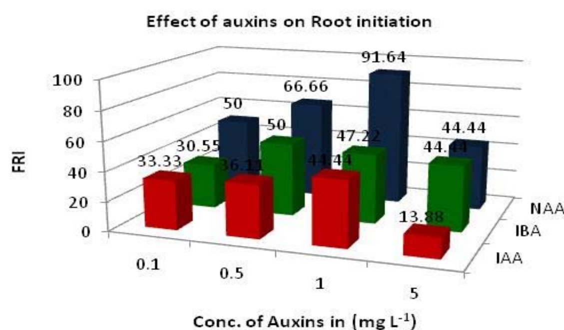
Graph 3: Effect of auxins on Root elongation (MRL) of *Hedychium coronarium*



Graph 2: Effect of additives on Shoot length (MSL) of *Hedychium coronarium*



Graph 4: Effect of auxins on Root number (MRN) of *Hedychium coronarium*



Graph 5: Effect of auxins on frequency of rooting (FRI) of *Hedychium coronarium*

DISCUSSION

Growth and regeneration of plants from *in vitro* tissue cultures can be improved by small amounts of some organic nutrients. Many of these amendments can be a source of amino acids, peptides, fatty acids, carbohydrates, vitamins and plant growth substances in different concentrations. The amount of these substances required for successful culture varies with the species and genotype.[30] Fortification of culture media with different plant growth regulators i.e. auxins and cytokinins is not enough to regenerate the plant with high efficiency. This type of cultures in some cases may be improved by incorporation of additives in the media due to their growth and development promoting activities.[31]

An adequate formulation of the nutrition media is essential to tissue culture, because media must supply the essential substances for *in vitro* growth development. Addition of activated charcoal on tissue culture media can be beneficial or adverse to growth and development. Activated charcoal as supplement on the media has been reported through the years by several authors due to its beneficial effects, such as, absorption of phenolic complex[16], root stimulator[32], rhizome growth[33], development, improvement and absorption of toxic substances present in the media.[13] It was showed that 2.0 gm L^{-1} of activated charcoal generated the best result on *in vitro* vegetative growth of *Cattleya walkeriana* plantlets.[34] Addition of active charcoal in culture medium may affect rooting. Activated charcoal in the medium culture improves not only rooting rates, but also roots growth of *Pinus pinaster.*[27] Similar results were reported by others authors.[35]

Positive effects of phloroglucinol on the stimulation of growth and shoot proliferation have been reported for some woody species[36] in terms of multiple shoot formation and axillary shoot proliferation.[37] PG has been reported to act synergistically with BAP in inducing early bud break and high frequency shoot proliferation of *Vitex negundo.*[25] An optimum concentration of 200 mg/L PG was reported as an optimum concentration to enhance shoot proliferation in coffee.[38] Among all the additives used PG and AC acted positively for multiple shoot regeneration from *in vitro* regenerated shoots. Whereas more conspicuous role of Phloroglucinol (PG.) in *H. coronarium* seems to be on the number of shoots induced (MSN) as well as shoot length (MSL).

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