



Simple and robust multiple shoot regeneration and root induction cycle from different explants of *Hypericum perforatum* L. genotypes

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Abstract

Hypericum perforatum L. commonly known as Saint John's Wort (SJW) is an economically important medicinal plant known for accumulating its valuable bioactive compounds in a compartmentalized fashion. The dark glands are very rich in hypericin, and translucent glands are filled with hyperforin. The antibiotic properties of the afore mentioned bioactive compounds make it hard to establish tissue regeneration protocols essential to put in place a transformation platform that is required for testing gene function in this challenging species. In this study, we report the establishment of a regeneration and root induction cycle from different types of explants. The regeneration cycle was set up for the continuous supply of roots and leaf explants for downstream transformation experiments. The most effective medium to obtain multiple shoot-buds from node cultures was MS (Murashige and Skoog, *Physiol Plant* 15:473–497, 1962) medium supplemented with 0.5 mg L⁻¹ 6-Benzylaminopurine (BAP) and 0.5 mg L⁻¹ indole-3-butyric acid (IBA). The same combination yielded copious amounts of shoots from root and leaf explants as well. For rooting the elongated shoots, MS medium devoid of plant growth regulators (PGRs) was sufficient. Nevertheless, addition of a low amount of IBA improved the quantity and quality of roots induced. Additionally, the roots obtained on a medium containing IBA readily developed shoot buds.

Key message

Rapid and multiple shoot regeneration from roots of *Hypericum perforatum* L. was established, which can be applied to downstream *Agrobacterium*-mediated transformation.

Keywords Multiple shoots · Leaf · Node · Root

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
CIM	Callus induction medium
DNA	Deoxyribo nucleic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
NaOCl	Sodium Hypochlorite

PGRs	Plant Growth Regulators
PPM	Plant protection medium
RIM	Root induction medium
SIM	Shoot induction medium
SJW	Saint John's Wort
v/v	Volume by volume
w/v	Weight by volume

Introduction

Hypericum perforatum L. is one of the top-selling medicinal herbs in the world. In most countries, the products are marketed as supplements. However, in the European community, products are available both as food supplements and as well manifested traditional drugs. According to market data from 2007 to 2008, more than 9.5 million packages of Saint John's Wort (SJW) were sold in Europe. At the same time, SJW products ranked 10th among top-selling herbal dietary

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supplements in the USA with a turnover of 8.1 million USD (Linde 2009). It is traditionally used for the treatment of mild depression symptoms (Laakmann et al. 1998, 2002; Wheatley 1998; Harrer et al. 1999; Melzer et al. 2010). The bioactive compounds responsible for this effect are hypericin, hyperforin, and other phenolics present in the plant (Müller et al. 1997; Butterweck et al. 2000). Hypericin is also used to detect bladder cancer (D'Hallewin et al. 2000; Sim et al. 2005) and in the treatment of cancer (Agostinis et al. 2002). In addition, the oil-based extracts of *Hypericum* species are known to possess anti-fungal properties (Cakir et al. 2005). Other pharmaceutical properties include anti-viral (Schinazi et al. 1990), neuroprotective (Silva et al. 2008), and diabetic wound-healing (Yadollah-Damavandi et al. 2015). Recently, it was demonstrated that various compounds isolated from *Hypericum* exhibited cytotoxicity against cancer cell lines, showed antibacterial (Feyzioglu et al. 2013) and anthelmintic activities as well as anti-HIV activity in infected MT-4 cells (Fobofou et al. 2015). Hypericin accumulates in specialized organs called dark glands (Mathis and Ourisson 1963; Robson 1981; Southwell and Campbell 1991; Fornasiero et al. 1998; Maffi et al. 2005; Zobayed et al. 2006; Kusari et al. 2015; Rizzo et al. 2019), whereas hyperforin accumulates in other structures called translucent glands (Soelberg et al. 2007). The dark glands are found in: margin of petals and leaves, sepals, pistils, anther tips and sometimes on stems of the plants but not on roots (Rizzo et al. 2020). The pale or translucent glands are located on leaves, sepals and petals (Ciccarelli et al. 2001). Despite a long history of traditional use and a wealth of knowledge about the bioactive ingredients, the characterization of genes and enzymes involved in their biosynthesis remains unclear. Moreover, information regarding the formation and differentiation of the glands that accumulate the bioactive products remains ambiguous (Rizzo et al. 2020). The glands in the placenta can reach a very high density compared to leaves (Rizzo et al. 2019). In addition, the differentiation of the dark glands in the placental tissue appears much later during development, which makes it possible to study the pre and post-developmental stages of dark glands. Metabolomics and transcriptomics data revealed novel compounds associated with dark glands as well as regulatory genes associated with the development of these organs. This led to identification of candidate genes associated with hypericin biosynthesis (Rizzo et al. 2019). The functional validation of these candidates, requires a robust regeneration system followed by a functioning transformation platform.

Agrobacterium-mediated transformation in *Hypericum* has proven to be very difficult (Hou et al. 2016). Increased production of xanthone and accumulation of active biological compounds were detected upon co-cultivation with *Agrobacterium* (Franklin et al. 2009) in *Hypericum* suspension cultures. Antibiotic properties of *Hypericum* extracts

against gram-positive bacteria, were reported previously (Schempp et al. 1999; Giudice et al. 2020), however, it was evident from our initial experiments that the growth of *Agrobacterium* used for transformation experiments is strongly repressed in the presence of *Hypericum* tissues. Similar problems were encountered in separate attempts at *Hypericum* transformation using *Agrobacterium* despite the utilization of different explants in large numbers (Franklin et al. 2007, 2008). Key genes involved in plant defense responses were investigated and were found to play an important role in *Agrobacterium*-mediated transformation in *Hypericum* (Hou et al. 2021). The same study suggested that dark glands of *H. perforatum* participate in defense responses by synthesis of secondary metabolites and defense proteins. As *Agrobacterium*-mediated transformation is still the most efficient method, we wanted to establish a protocol that involves an explant that does not contain glands that accumulate hypericin or hyperforin. In general, reproducible protocols for a routine in vitro regeneration of different *Hypericum* spp. is lacking in the current literature. Existing protocols involve direct or indirect regeneration from shoot tips (Abdollahpoor et al. 2017) or leaf explants (Pretto and Santarém 2000), which may not help with *Agrobacterium*-mediated transformation, as they contain the dark glands. Goel et al. (2009) showed shoot regeneration from root cultures. Nonetheless, these reports involved the usage of high amounts of plant growth regulators (PGRs). The main aim of our study was to increase the possibility of obtaining roots in sufficient quantity for downstream experiments. As roots do not contain dark glands, they might respond better to *Agrobacterium*-mediated transformation. This robust multiple shoot regeneration protocol will also allow us to obtain leaves prepared in such a way as to avoid dark glands.

Materials and methods

Plant material

We used a *Hypericum perforatum* genotype collection grown in the greenhouse and fields of IPK-Gatersleben. For germination studies, seeds were collected from the field-grown genotypes HyPR-01, HyPR-05, H06-1540, -1656, -1662, -2574, -2720, -2886, -2925, -3160, -3197, and 3233. For obtaining the seeds, dried inflorescences were collected, seeds extracted from their capsules, and dried under the sun. These seeds were stored at room temperature for immediate use or kept at 4 °C for long term storage. Standardization of seed sterilization and germination was done using genotype HyPR-01 seeds and was used as wild type control, as this genotype produced bountiful amount of seeds. Moreover, this genotype is well studied for its genomic and metabolic background (Rizzo et al. 2019). For experiments involving

nodes, leaves and roots, greenhouse grown genotypes, HyPR-05 and KEW-Hyp-37 and seed-grown VitaPlant 1.0 were used. For these experiments too, HyPR-01 seedling derived explants were used as wild-type control.

Sterilization

Seeds were treated with 70% (v/v) ethanol containing 0.1% (v/v) Tween-20 for 6 min, washed in sterile water 3 times or until the foam disappeared. These seeds were surface sterilized using 12% (v/v) NaOCl containing 0.1% (v/v) Tween-20 for 20 min. NaOCl was washed off using sterile water 3 to 4 times or until the foam vanished.

For sterilization of node explants, stem cuttings collected from the greenhouse, were immediately immersed in Millipore water. The cuttings were spread on a Petri-dish (still immersed in water) and the leaves were carefully removed using a scalpel. The stem was cut into smaller pieces (3 to 4 nodes). These nodes were transferred into a 50 ml sterile Falcon tube. From here on all the steps were conducted under a Laminar Air Hood. A quick rinse was given in 70% (v/v) ethanol and the nodes were washed in sterile Millipore water. The nodes were then sterilized using 0.25% (v/v) NaOCl containing 0.01% (v/v) Tween-20 for 2 min. NaOCl was washed off using sterile Millipore water 3 to 4 times or until the foam disappeared.

Seed germination and further growth

Seeds were sterilized as explained above and were briefly dried on sterile tissue paper and were cultured (50 seeds per Petri-plate) on MS (Murashige and Skoog 1962) medium without any growth regulators. Up to 100 seeds per experiment were used. These Petri-plates were covered in aluminum foil and were incubated at 4 °C for 4 days before they were opened and introduced into the standard culture conditions of 26 ± 2 °C at 16 h photoperiod ($50 \mu \text{mol m}^{-2} \text{s}^{-1}$) with 50% humidity. Resulting seedlings at cotyledonary stage were sub-cultured upright on to MS basal medium. All the cultures explained in this study were maintained at standard culture conditions unless otherwise mentioned.

Shoot multiplication and rooting of shoots from seedling explants

For multiple shoot induction, the seedlings of HyPR-01 were either used as such or the nodes were excised and re-cultured on MS medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP); 0.1 to 1.0 mg L⁻¹ and indole-3-butyric acid (IBA); 0.01 to 1.0 mg L⁻¹ (SIM). The same range of IBA concentrations (RIM) was used for rooting experiments using nodes and shoots from seedlings. All media used in this study had basal

MS salts and vitamins, 3% (w/v) sucrose, solidified with 0.75% plant agar. The pH of the media was adjusted to 5.8 prior to sterilization by autoclaving at 121 °C for 20 min. All the filter-sterilized PGRs were added before dispensing in culture vessels or Petri-plates.

Cultures from node explants from the greenhouse-grown plants

It was necessary to standardize a platform for in vitro cultures from explants collected from the vegetatively propagated plants maintained in the greenhouse to ensure the genetic integrity of the explants to be used in transformation experiments. For multiple shoot induction and from node explants, after sterilization, the stem pieces were cut on sterile tissue paper, to isolate the nodes with a minimum of 0.5 cm below and up to 0.4 cm on the top. This difference in the internodal region helped in orienting the explants on the medium. The nodes were cultured upright on MS medium containing 200 $\mu\text{L L}^{-1}$ of PPM (Plant Protection Medium; Plant Cell Technology, USA). Once the axillary bud sprouted, the nodes were sub-cultured on MS medium supplemented with the best concentrations and combinations of BAP and IBA from the above experiments for multiple shoot proliferation. Once contamination free cultures were obtained, further cultures were done on medium devoid of PPM. Observations were made every 15 days up to 60 days.

Multiple shoot induction on root explants

Healthy and fresh roots from the in vitro grown shoots cut into 5 cm long pieces were cultured on multiple shoot induction medium used in node culture experiments. Roots were also cultured in liquid MS media, with same PGR combination and concentration which was incubated in standard culture room conditions (see seed germination section) and 100 rpm. Once the shoot induction started, these roots along with shoots/buds were divided into small pieces and were cultured on solid medium. As and when the shoots multiplied, they were further divided into smaller clusters and sub-cultured every 15–20 days on the same media combination for further proliferation.

Callus induction and shoot regeneration from root and leaf explants

Callus induction and shoot regeneration from callus were attempted from root and leaf explants. Auxins 2,4-D (2,4-Dichlorophenoxyacetic acid), IBA and NAA (1-Naphthaleneacetic acid) alone (0.1 to 0.5 mg L⁻¹) (CIM) and in combination with BAP (0.5 and 1.0 mg L⁻¹) were used. In vitro grown shoots were utilized for these experiments. Leaf explants were cut in particular way to obtain different

types of tissues. (1) The leaf was cut transversely into three portions (2) a square was cut out from the middle avoiding all the dark glands at the margin. The cultures were incubated under light as well as dark conditions for callus initiation and shoot regeneration. Observations were made after 10 and 30 days.

Shoot elongation and rooting

Multiple buds induced on nodal, root and seedling explants were divided into small clusters and were cultured on MS media devoid of PGRs for elongation. Elongated shoots were again divided into smaller clusters and were sub-cultured onto MS basal media for further growth. Once the shoots reached a height of 3–4 cm, the individual nodes/shoot tips were harvested for rooting. Root induction was initiated either on MS basal media or on MS media supplemented with 0.5 mg L^{-1} IBA (RIM). These experiments were conducted as a routine cycle: (1) *in vitro* shoot culture (2) multiple axillary bud induction (3) elongation of shoots (4) rooting of elongated shoots (5) multiple shoot induction from roots, and (6) rooting of these shoots (Fig. 1) for the continuous supply of explants for further studies.

An overview of MS medium supplemented with different combinations and concentrations of PGRs used for standardization of all the methods used in this study are given in Table 1. The working combinations and concentrations are given in red.

Ploidy measurements

Ploidy of plant material derived from seed germination (seedlings) or cuttings (leaves) was measured using a Partec CyFlow flow cytometer equipped with a green laser (532 nm) for the detection of fluorescence derived from Propidium Iodide (PI). Plant material was chopped for 30–40 s with a razor blade in 300 μl of PI buffer. After chopping, 700 μl of PI buffer were added and the entire sample was filtered using 30 μl Partec CellTrics filters. The formula for the PI buffer is provided in Table S1 (Supplemental Data).

Statistical analyses and figures

Results were analyzed statistically using RStudio (version 4.1.2). $p < 0.05$. One way and two-way ANOVA, Standard error and post hoc analyses were conducted using packages: ggplot, car, readxl, dplyr, and multcomp. Figures were edited and figure-plates were created using GIMP version 2.10.20.

Results

Seed germination

Seed germination in HyPR-01 was achieved without any contamination MS medium devoid of PGRs. Radicles emerged within 5 days of incubation in the culture room. Within 2 weeks we obtained seedlings that had developed enough for harvesting leaves and nodes used in downstream experiments. The same sterilization method, when used for other genotypes mentioned in the materials and method section, they responded in a similar fashion and the results are as shown in Fig. 2. Sub-culture of seedlings at cotyledonary stage was necessary for proper shoot and root development. The seedlings reached an average height of 3.5 cm with 5–6 nodes within 30 days of germination. In our experiments, a minimum of 67.9% (HyPR-05) to a maximum of 100% (H06-1662 and H06-3233) normal germination phenotypes were obtained independent of the genotype used (Fig. 2).

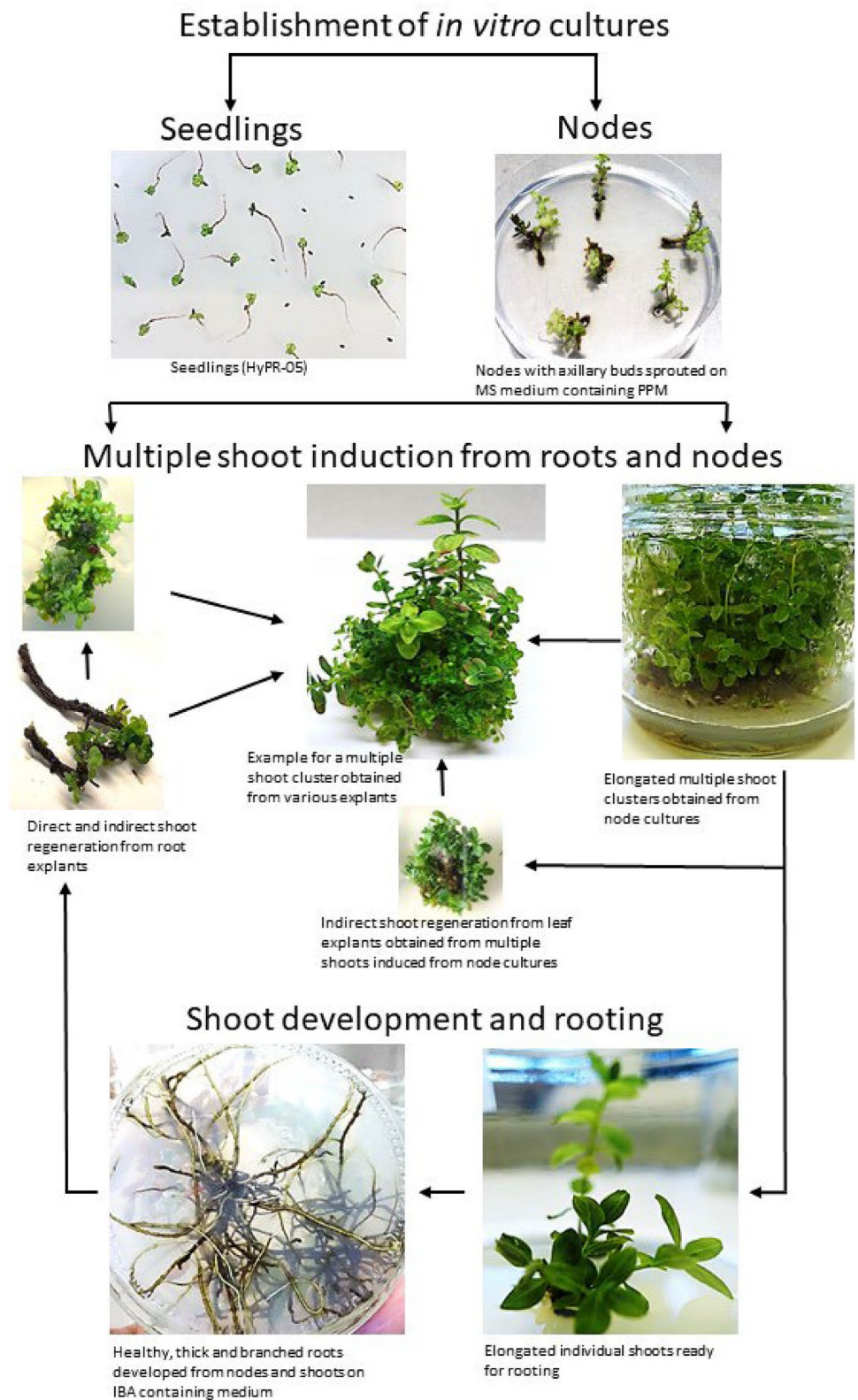
Shoot multiplication and rooting

The seedlings consisted of a straight growing shoot with no or sporadic branches. The roots revealed a similar non-branching phenotype (Fig. 3A, B). To increase the body mass of the seedling, the shoot was cut into nodes and cultured on MS medium augmented with BAP alone and in combination with IBA. When BAP alone was used, it increased the number of shoots up to an average of 4% explant. However, the leaves were brittle and the shoots did not elongate or develop any roots on the same media (Fig. 3C). The cluster of shoots obtained on the BAP containing medium readily elongated on MS medium without any PGRs. Random root formation of up to 10% was observed on the nodes/shoot tips cultured on MS medium. However, the results were not consistent. On MS medium containing 0.5 mg L^{-1} IBA, 92.5% of the shoot tips and nodes rooted and branched well (Table 2). A maximum of 15 to 18 roots were developed per shoot (Fig. 3D).

In vitro cultures of node explants from greenhouse-grown plants

Due to availability of abundance of seed material and easy to grow conditions all the initial experiments and standardizations were done using HyPR-01 seedlings. However, to avoid any genetical variation due to free pollination, and to provide homogenous material for the intended downstream transformation experiments, it was necessary to use vegetatively propagated and maintained plants in the greenhouse.

Fig.1 Schematic representation of regeneration cycle in vitro for *H. perforatum* genotypes. Arrows indicate the possibilities of developing alternate ways to produce explants and rooted shoots

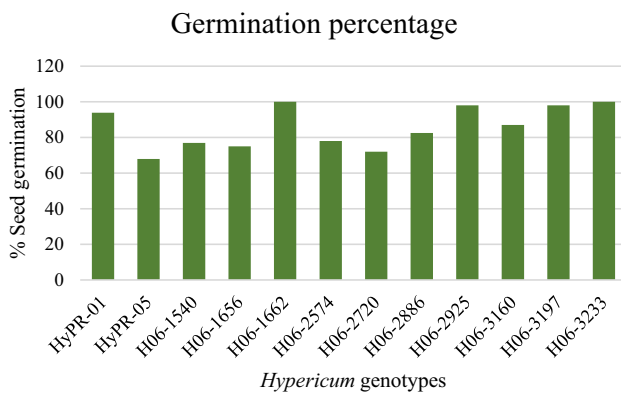


Ploidy characterization of the used material showed that plants derived from seed germination after free pollination, show heterogeneous ploidy levels (Fig. S1). On the other

hand, vegetatively propagated material was characterized by homogeneous ploidy levels (Fig. S2 and S3).

Table 1 Overview of Media and PGR concentrations and combinations used in this study. The working combinations and concentrations are given in red

Purpose	Medium	BAP (mg L ⁻¹)	IBA (mg L ⁻¹)	NAA (mg L ⁻¹)	2,4-D (mg L ⁻¹)
Seed germination	MS	0	0	0	0
Multiple shoot regeneration from nodes (SIM)	MS	0.0, 0.5, 1.0, 1.5	0.1, 0.3, 0.5, 0.8, 1.0	0	0
Root induction medium (RIM)	MS	0	0.0, 0.01, 0.05, 0.08, 0.1, 0.3, 0.5, 0.8, 1.0	0	0
Callus induction (CIM)	MS		0.1, 0.3, 0.5, 0.8, 1.0	0.1, 0.3, 0.5, 0.8, 1.0	0.1, 0.3, 0.5, 0.8, 1.0
		0.5, 1.0	0.1, 0.3, 0.5, 0.8, 1.0	0.1, 0.3, 0.5, 0.8, 1.0	
				0.1, 0.3, 0.5, 0.8, 1.0	

**Fig. 2** Germination efficiency of seeds from different genotypes of *Hypericum perforatum* L. on MS medium

The nodes collected from the green-house grown plants cultured on MS medium containing PPM took 7 to 8 days to sprout the axillary buds. Once the axillary buds developed up to 1.5 cm long, and looked contamination free, they were sub-cultured onto MS medium devoid of PPM. This was important as leaving them on PPM containing medium for a longer period of time retarded the growth in general and chlorosis was observed on a few genotypes. The cut ends of the nodes taken from the greenhouse turned brown or black in color, most likely due to phenolic oxidation. It was necessary to remove this portion during sub-culture to make sure that the explants received proper nutrients. On MS medium, the axillary buds continued growing without developing any side branches or multiple shoots. Sub-culture of these nodes with sprouted axillary buds onto SIM (Fig. 4A and Fig. 5A–C) produced multiple shoots. These shoot clusters elongated on MS medium devoid of PGRs. Once elongated to 3–5 nodes, they were rooted on RIM containing medium (Table 1).

The highest response percentage and number of roots were observed on media containing 0.5 mg L⁻¹ IBA. Similar to in vitro developed shoots and nodes, green-house grown

node explants cultured on basal MS media devoid of PGRs also occasionally developed roots at low numbers, but the frequency of root development was low (up to 10%). Even though these roots were in general thick and unbranched, there were also roots with sparse branching (Fig. 3A). MS media containing IBA initiated root development within 5 to 10 days and by 15 days reached an average of 2 cm. These roots appeared healthy, branched and were fast-growing.

These results were applied to other genotypes viz., HyPR-05, Vitaplant 1.0, KEW-Hyp-37. The percentage response of these genotypes maintained a similar pattern for the PGRs used in this study. However, the best response thus far was exhibited by genotype HyPR-05. Starting with an initial 24 nodes, 24 culture jars full of healthy shoots were obtained within 120 days. KEW-Hyp-37 and Vitaplant 1.0 also showed a similarly high number of multiple shoot formation. From an initial 20 and 2 nodes respectively, they produced 15 and 7 jars full of shoots producing an average of 300 shoots per node.

Shoot regeneration from roots

Healthy-looking and vigorously growing thick roots (Fig. 6A) obtained on IBA-containing medium were chosen for regeneration experiments. Very thin roots did not develop any shoot buds (Fig. 6B). The thicker the roots, the healthier were the shoot-buds and shoots developed from them. The root pieces developed shoot buds cultured on basal MS medium, BAP alone, and in combination with IBA (Fig. 4B). The roots started shoot bud initiation within 10 to 15 days. Most of the time, the roots were covered in shoot buds to give an appearance of a lichen-covered thin branch (Fig. 5D). At this stage the roots were divided into smaller pieces and were sub-cultured on MS medium. On basal MS basal medium shoots elongated faster than on the PGR containing medium. The root pieces cultured on BAP-IBA combination developed numerous short shoots, giving the appearance of a moss bed (Fig. 5E). Dividing the roots into

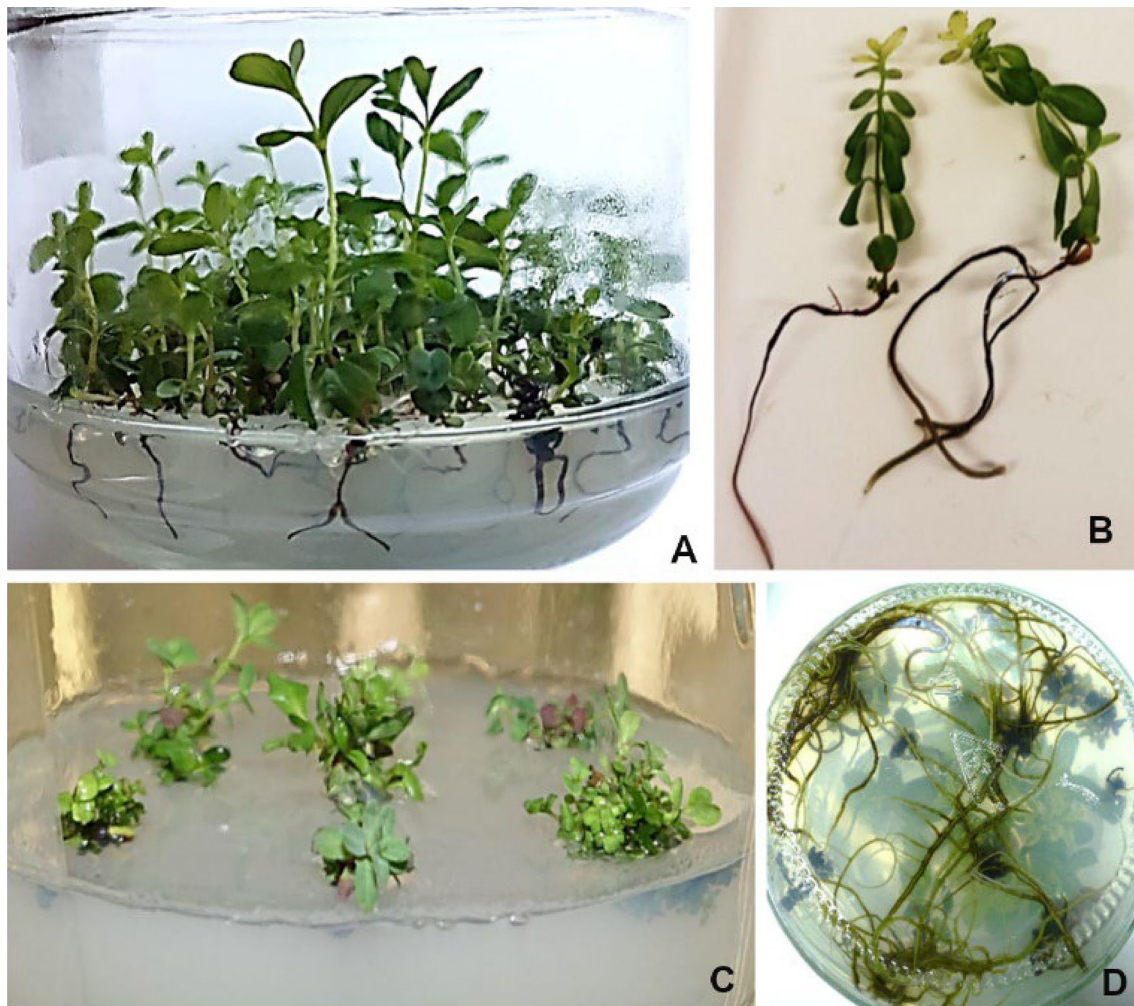


Fig. 3 Shoot multiplication and rooting from seedling explants: **A, B** Seedlings showing a single straight shoot and un-branched or sporadically branched roots **C** Nodes from seedlings on BAP containing MS medium **D** Rooting of elongated shoots on MS medium supplemented with IBA

Table 2 Rooting of nodes and shoot tips from seedlings

IBA Concentration (mg L ⁻¹)	Percentage of responding nodes (%)	Av. no. of roots/node ± Std. Err	Response percentage of seedling shoots	Av. no. of roots/seedling shoot ± Std. Err
0.0	10	1.3 ± 0.152 ^e	53	3.5 ± 0.31 ^f
0.01	30	1.4 ± 0.22 ^e	90	3.7 ± 0.26 ^f
0.05	33.2	2.0 ± 0.15 ^{de}	72.72	5.7 ± 1.57 ^{d,e}
0.08	35	2.9 ± 0.26 ^d	100	5.3 ± 1.14 ^{d,e,f}
0.1	56	4.2 ± 0.42 ^c	95	7.2 ± 0.53 ^d
0.3	55	3 ± 0.29 ^d	98	12.9 ± 0.48 ^c
0.5	85	10.3 ± 0.7 ^a	100	18.7 ± 0.45 ^a
0.8	72	10.8 ± 0.46 ^a	100	16.2 ± 0.6 ^b
1.0	75	5.6 ± 0.45 ^b	100	15.5 ± 0.3 ^b

Values followed by different alphabets are significantly different within the two sets of data on nodes and shoots

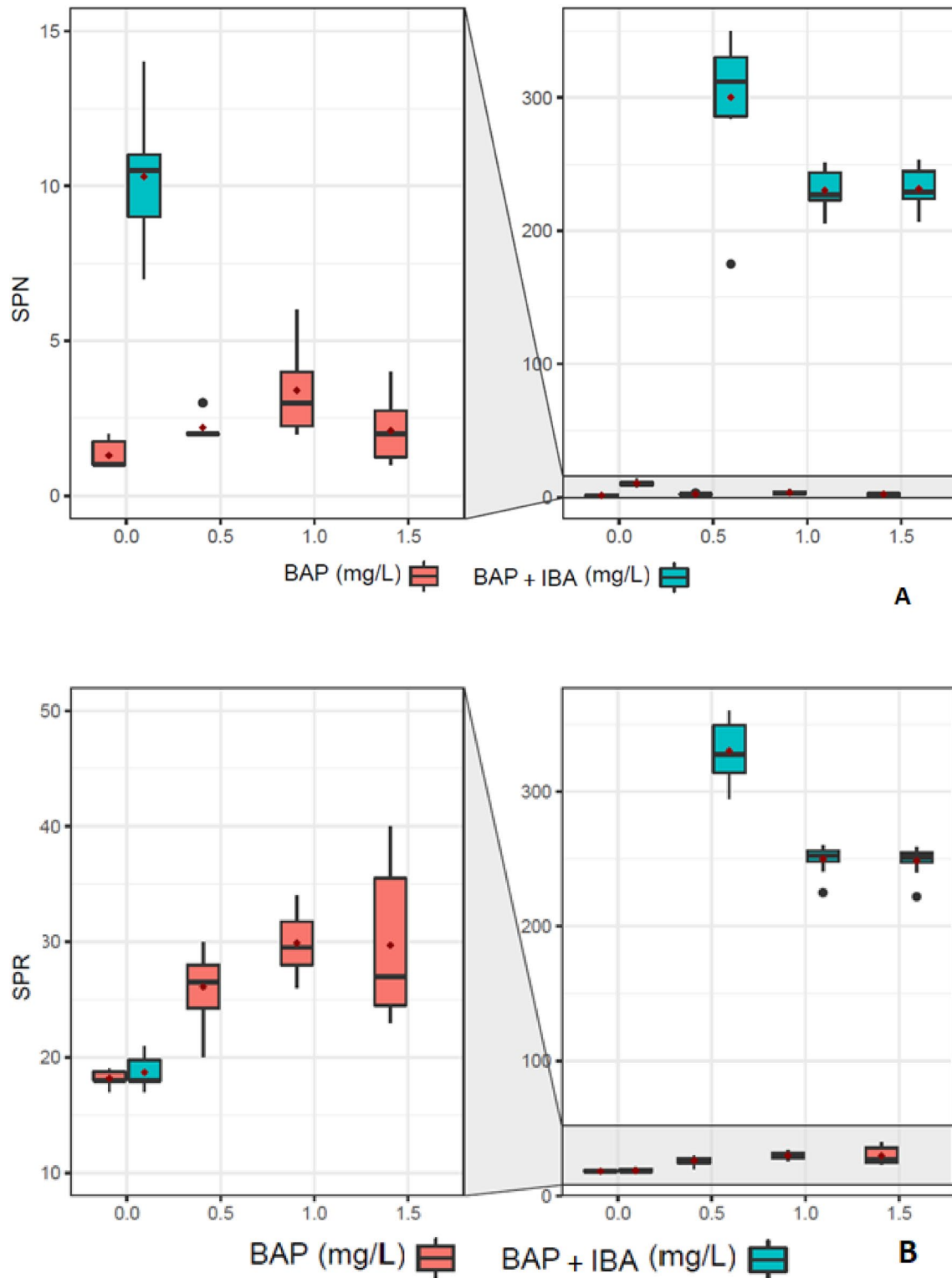


Fig. 4 Boxplots of average number of shoots obtained on node and root explants on MS media supplemented with different concentration (in mg L^{-1}) and combination of PGRs. **A** Number of shoots devel-

oped on node on SIM **B** Number of shoots developed on root explant on SIM. *SPN* Shoots per node, *SPR* shoots per root

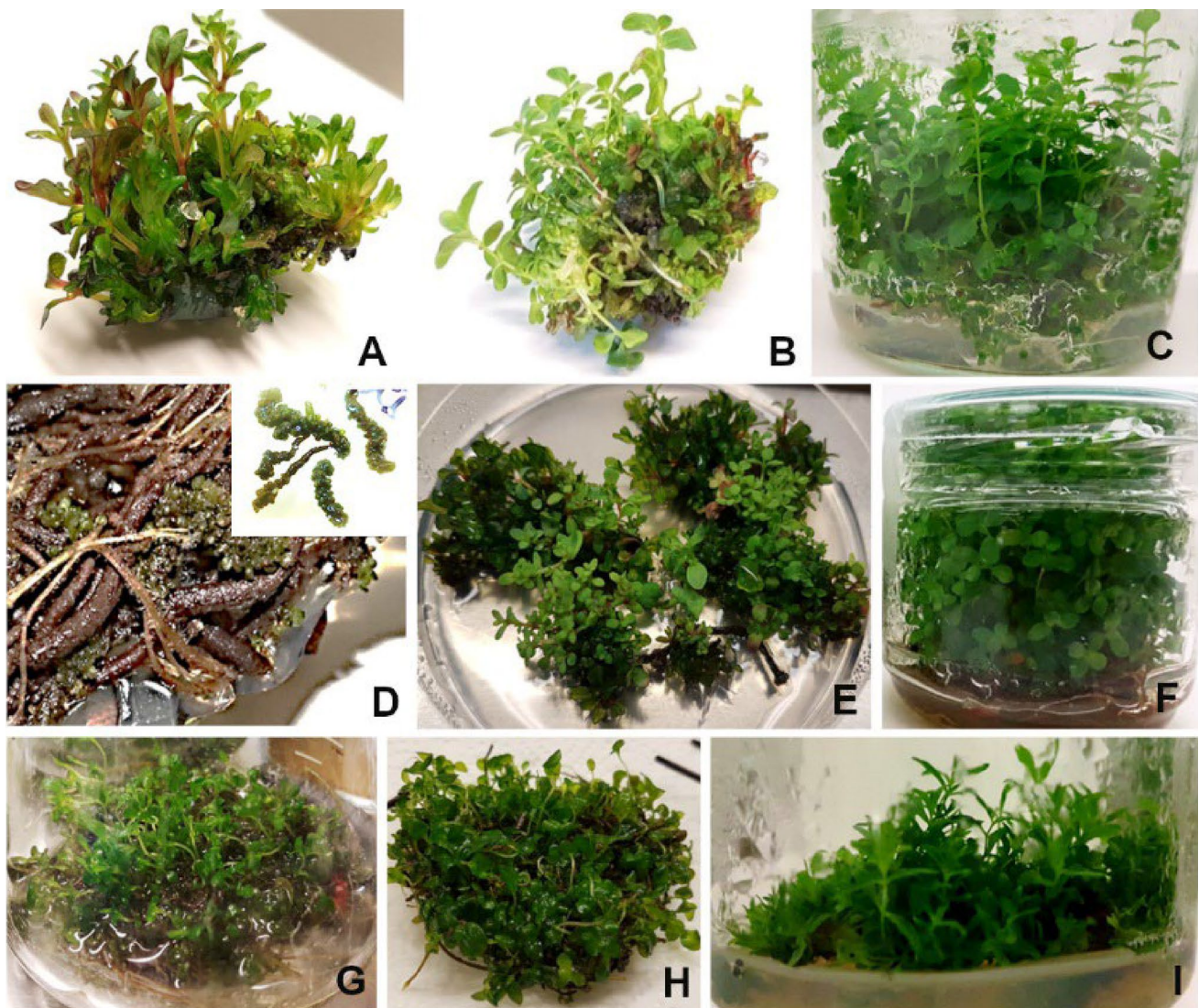


Fig. 5 Multiple shoot regeneration from node and root cultures **A, B, C** Regeneration from node **D, E, F** from roots **G, H, I** from root liquid culture

clusters and sub-culturing them onto MS medium helped in further proliferation of shoots similar to the clusters developed from the nodal explants. With every sub-culture, number of elongated shoots increased (Fig. 5F). Elongated shoots when moved for rooting on 0.5 mg L^{-1} IBA, where they easily rooted and became ready for the next cycle. A maximum of 330.3 shoot buds were obtained from 3 cm of root. Similar results were obtained from roots cultured in liquid medium (Fig. 5G–I). Comparable results were obtained from genotypes HyPR-05, Vitaplant 1.0, and KEW-Hyp-37.

Shoot regeneration from callus

On all concentrations of auxins used, smooth calli were obtained from all the root explants within 2 weeks. There

was little influence of light on calli cultures except that the cultures grown under dark conditions were paler in color in comparison to those calli grown in the light. Under the influence of IBA and NAA at lower concentrations (from 0.1 to 0.3 mg L^{-1}) only a few stand-alone root initiations from stem explants occurred. However, 2,4-D initiated roots along with calli only at a higher concentration of 0.5 mg L^{-1} . These calli did not develop further on the same media or when sub-cultured on media containing auxins and cytokinins alone or in combination. These conditions were therefore not considered for any further experiments. Based on our aforementioned experience with the efficiency of IBA in combination with BAP, and that NAA behaved similar to IBA, we determined that further experiments would utilize only these two auxins. As the amount of callus produced

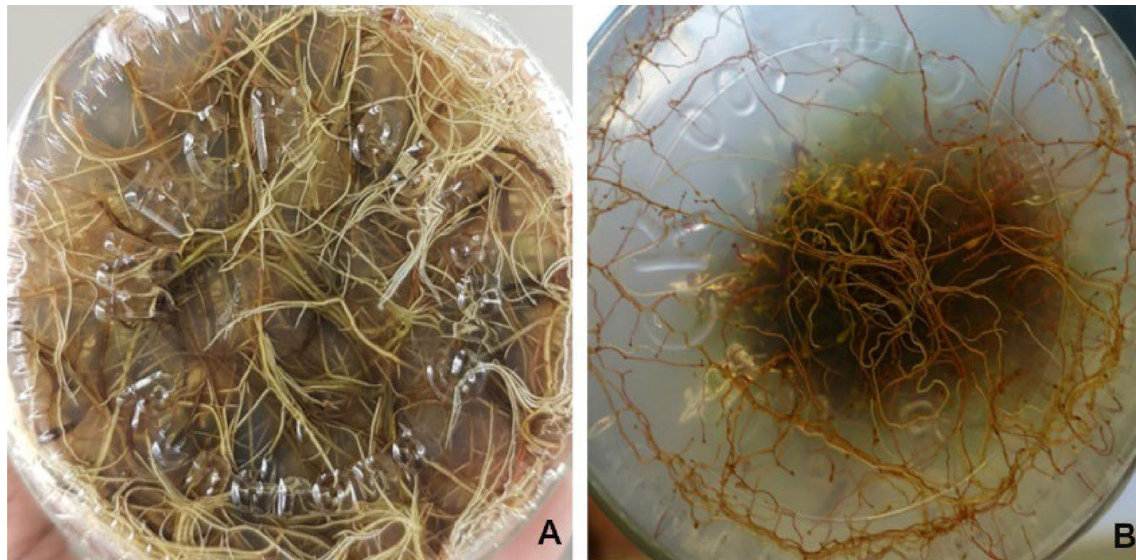


Fig. 6 Type of roots of *Hypericum perforatum* **A** Healthy thick roots which readily gave shoots on appropriate medium and **B** Roots which were not suitable for quick regeneration

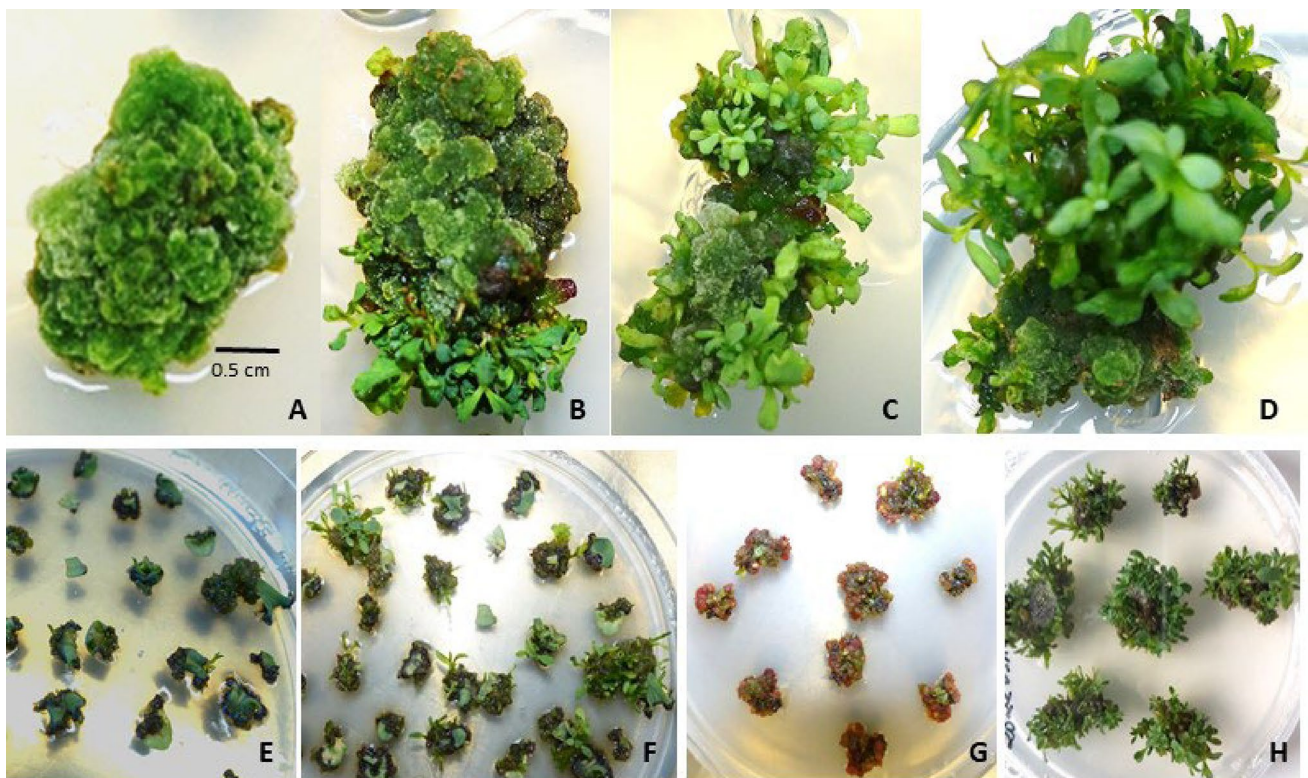


Fig. 7 Shoot regeneration from calli **A–D** Callus developed on root explants and regeneration of shoots **E** and **F** Leaf portion with dark glands after 10 and 30 days of incubation on CIM **G** Middle portion

of the leaf without dark glands, 30 days after culture **H** Calli with shoot buds cultured on MS medium proliferating shoots in 15 days from glandless leaf portion

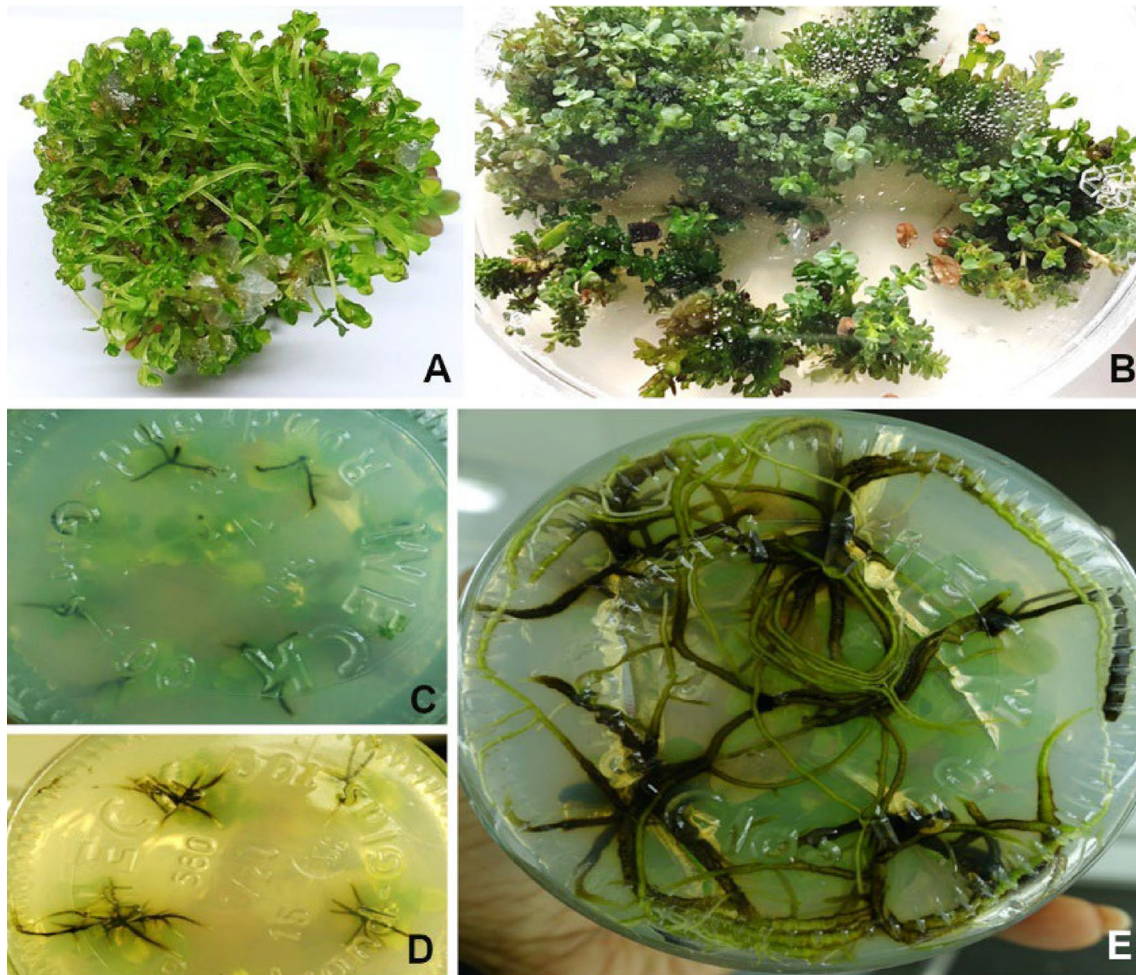


Fig. 8 Shoot elongation and rooting **A** Glassy brittle shoots formed under the media **B** Healthy clusters of elongating shoots upon subculturing the glassy shoots **C** Roots obtained on MS media **D** and **E**

Roots developed on MS media containing 0.5 mg L^{-1} IBA after 10 and 30 days respectively

did not change at lower or higher concentrations of supplemental auxins, we chose 0.1 mg L^{-1} along with either 0.5 or 1.0 mg L^{-1} BAP. For this experiment, only leaf and root explants were used. On root explants, green compact calli were formed on media containing 0.5 mg L^{-1} BAP in combination with 1.0 mg L^{-1} of either IBA or NAA (Fig. 7A). On higher BAP containing medium, the amount of callus was much less and there was a tendency to develop shoot buds. When the green compact calli were cut into ca 1 cm pieces and cultured on shoot-bud induction medium, up to 50 shoot buds developed (Fig. 7B, C).

Nodular calli with shoot buds and root buds were formed at the cut ends of leaf explants. The number of bud structures depended on the cut end area of the explant. Approximately 30 buds were obtained on an explant with only one cut end (Fig. 7E), and a maximum of 87 buds were obtained on an explant with four cut ends (Fig. 7G). It did not make any difference if the sample contained dark glands (Fig. 7F, G).

Similar results were observed on explants incubated under dark or light conditions. More root buds were seen on media containing low BAP. When grown on high BAP containing media, shoot-buds sprouted within 10 days. Once the shoot buds were set, calli from both types of explants were transferred to MS medium devoid of PGRs for shoot elongation (Fig. 7D, H). Elongated shoots rooted comfortably on MS medium containing IBA. Callus regeneration was not tried for other genotypes.

Shoot elongation and rooting cycle

Irrespective of the genotype of the explants used, node cultures, leaves, roots and calli on roots developed multiple shoots on MS medium containing BAP and IBA. The shoots were short and compactly arranged. We observed many shoots branching off into the media, which became glassy and brittle (Fig. 8A). Nevertheless, when divided into

small clusters of shoots/shoot buds and sub-cultured onto MS basal media, they grew healthier within 3 weeks of culture (Fig. 8B). The elongated shoots from MS basal medium were cultured on IBA containing medium for rooting. On MS, even though few roots developed, they did not look healthy or branched (Fig. 8C). On IBA supplemented MS medium, the roots developed faster, stronger and branched (Fig. 8D, E). The culture cycle included shoot initiation on MS medium, multiplying the shoot numbers on BAP and IBA containing medium, elongating the obtained shoot-buds on MS basal medium, and rooting the elongated shoots on MS media containing IBA. This cycle was multifaceted and explants could be taken from any stage of the cycle (Fig. 1).

Discussion

Hypericum spp. is traditionally used as medicinal plant and has a high commercial value all over the world. The future of *Hypericum* research is highly dependent upon the ability to manipulate candidate genes involved in multiple biosynthetic and developmental processes. Success of these future endeavors is predicated upon having a stable, reliable protocol for the regeneration of explants. The efficiency of direct and indirect regeneration in *Hypericum* was shown earlier (Cellarova et al 1992; Brutovska et al. 1994; Cellarova and Kimakova 1999; Bernardi et al. 2007) and the effectiveness varied greatly among types and concentrations of PGRs used and also depended on the species and genotype. As first step, genotype HyPR-01 was used for initial experiments and standardization due to its well-studied genomic and metabolic background (Rizzo et al. 2019). Furthermore, this genotype produced exuberant amount of seeds when allowed to pollinate freely in the field conditions. For these reasons, all initial standardization experiments were conducted using the seedlings. On PGR free MS medium, we obtained from 68 (HyPR-05) to 100% (H06-1662 and H06-3233) germination within 5 to 7 days after they were incubated under normal culture conditions explained in the materials and methods section which was in contrast to a 50% normal phenotypes obtained by Cardoso and Oliveira (1996). Seeds obtained after free pollination germinated into seedlings which were not true to type due to different ploidy levels and reproductive pathways present in the species (Molins et al. 2014). This was true for our studies too (Supplementary Figs. 1 and 2). Hence we looked for alternative explants for regeneration studies.

Most of previous studies revolved around regeneration from seedlings, leaves, nodes or shoot tips which contain dark glands which do not coincide with our aim of producing materials suitable for *Agrobacterium*-mediated transformation. In our studies, we found that BAP was very effective at a 0.5 mg L⁻¹ in inducing a large number of shoots in

combination with IBA (SIM). This corroborates with the results observed on the effectiveness of BAP compared to other PGRs alone or in combination used by other authors (Cellarova et al 1992; Brutovska et al. 1994; Cellarova and Kimakova 1999; Franklin and Dias 2006; Bernardi et al. 2007; Işıkalan et al. 2011). However, the basal media used for establishing in vitro cultures in the past were different viz., Linsmaier and Skoog (1965) in combination with Gamborg et al. (1968) vitamins (Cellarova et al. 1992), MS in combination with B5 vitamins (Brutovska et al. 1994). While BAP was used in combination with kinetin for producing shoots in large numbers (Cellarova et al. 1992, Işıkalan et al. 2011), in our studies we found that BAP in combination with IBA was most effective in producing shoots in large numbers in all the genotypes tried (up to or above 300 shoots). Like in studies by Işıkalan et al. (2011) on *H. spectabile*, we also obtained shoot regeneration very smoothly on MS basal medium. Syahid and Wahyuni (2019) showed that rosette formation can be avoided by the addition of silver nitrate in prolonged cultures. However, in our cultures, we did not find any rosette formation. Occasional drying of shoots were observed even though fresh and healthy materials were used in the cultures but the number of drying shoots were not significant to be considered problematic.

Rooting of shoots obtained from any starting material, in our studies was obtained on medium containing IBA. A similar effect of IBA on rooting was also observed by Abdollahpoor et al. (2017). Effectiveness of IAA on rooting was demonstrated on different species by Oluk and Orhan (2009), Işıkalan et al. (2011). Similar to our findings, rooting was observed also on MS medium devoid of PGRs by Işıkalan et al. (2011) and Syahid and Wahyuni (2019). However, in our studies we observed that the roots formed were not healthy or branched and they grew very slowly compared to the ones on IBA containing medium.

Summary and conclusion

This report establishes a protocol that opens the possibility for an accelerated program in *Hypericum* molecular studies. *H. perforatum* root cultures could be quickly initiated using the described regeneration cycle. Flowering fresh plants or dried aerial parts are used as basic material for most of the drug preparations (EMEA Report 2009). The quality of the raw plant material plays a significant role in maintaining a high and constant quality. Climate conditions, soil type (Krasteva et al. 2013), soil containing heavy metals (Ullah et al. 2012), ecological effect, genetic factors (Cellarova et al. 1997; Kosuth et al. 2003), timing of harvest (Kaçar et al. 2008), method of drying influence the quality and content of the raw material. Establishing a vigorous in vitro regeneration system can also help circumvent this

problem, as it can provide homogenous plant material for the manufacture of oil extracts and natural products. Moreover, tissue-culture grown materials have much higher yields of phytochemicals, which can offset the production cost for quality content (Kirokosyan et al. 2000, 2003). A simple, efficient and repeatable regeneration cycle demonstrated here could be applied to other species of *Hypericum*. Toki et al. (2006) indicated in their studies on rice that freshly de-differentiating cells are more amenable for transformation than intact tissues. A similar opinion was also shared by (Kuta and Tripathi 2005). This method of regeneration in *Hypericum* might also help alleviate the problem of recalcitrance to *Agrobacterium*-mediated transformation by providing an alternative explant which is amenable to genetic manipulations.

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Declarations

Competing interest The Authors declare no financial or non-financial conflict of interest related to this work.

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