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Micropropagation, metabolite profiling, antioxidant activities and chromatographic determination of bioactive molecules across *in vitro* conditions and subsequent field cultivation stages of 'Shampoo Ginger' (*Zingiber zerumbet* L. Roscoe ex Sm)

Jaykumar J. Chavan[a,b,*], Parthraj R. Kshirsagar[c], Sandeep R. Pai[d], Nilesh V. Pawar[e]

[a] Department of Botany, Yashavantrao Chavan Institute of Science, Satara 415 001, India
[b] Department of Biotechnology, Yashavantrao Chavan Institute of Science, Satara 415 001, India
[c] Department of Biotechnology, Shivaji University, Kolhapur 416 004, India
[d] Amity Institute of biotechnology (AIB), Amity University, Mumbai-Pune Expressway, Bhatan, Post, Somathne, Panvel, Mumbai 410 206, India
[e] Department of Botany, The New College, Kolhapur 416 012, India

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ABSTRACT

Zingiber zerumbet (L.) Roscoe ex Sm, an aromatic rhizomatous herb widely used in food, beverages, medicine and for ornamental purposes. It was considered as spice ginger until the discovery of zerumbone (a monocyclic sesquiterpene), curcuminoids (a group of polyphenolic compounds) and other bioactive molecules. These compounds have great demand due to their nutritional, cosmeceutical and pharmaceutical properties. We investigated an efficient *in vitro* regeneration system for large-scale propagation and determination of metabolites accumulated under *in vitro* and subsequent field cultivation stages of *Z. zerumbet*. The maximum numbers of shoots (13.0 ± 0.9) as well as roots (43.0 ± 2.8) were obtained when rhizome bud explants were cultured on Murashige and Skoog's (MS) medium supplemented with 6-benzylaminopurine (4.0 mg/l) and indole-3-butyric acid (1.0 mg/l). Micropropagated plantlets were transferred to soil and successfully established in the field. Polyphenolic profile, antioxidative properties and RP-HPLC determination of pharmaceutically important molecules *viz*. zerumbone and curcuminoids were evaluated from various *in vitro* micropropagation and subsequent field cultivation stages. The results of the field trials showed that, micropropagated plants accumulated higher levels of metabolites, antioxidants activities as well as bioactive molecules after 9 months of field cultivation and retained stability in all biochemical parameters till dormancy. Thus outcome of these studies provides a view of the current state of research on the micropropagation, proper harvesting stage, enhanced and stable supply of metabolites, antioxidants and bioactive molecules *i.e.* zerumbone and curcuminoids.

1. Introduction

Zingiber zerumbet (L.) Roscoe ex Sm. (family- Zingiberaceae), commonly known as shampoo ginger, bitter ginger or pinecone is an aromatic and rhizomatous herb which grows naturally in the damp shaded areas or mountain slopes (Koga et al., 2016). This herbal plant is believed to be native to India and is cultivated as spice and also for medicinal purposes (White, 2007; Chang et al., 2012). In India, it occurs naturally in certain localities of the Himalaya and the Western Ghats, two mega-biodiversity centers. It is commonly used in folk medicine, as food condiments, and for ornamental purposes (Wagner et al., 1990; Yob et al., 2011).

Shampoo ginger is a rich source of different classes of compounds that belong to a wide variety of chemical metabolites including polyphenols, alkaloids and terpenes (Matthes et al., 1980; Jang et al., 2004; Chung et al., 2007; Dai et al., 2013). Over hundred chemical constituents of varied metabolite groups have been reported from *Z. zerumbet* (Ruslay et al., 2007; Dai et al., 2013; Koga et al., 2016). Due to

Abbreviations: AEAC, Ascorbic acid equivalent antioxidant capacity; DMPD, N, N-dimethyl-p-phenylenediamine; DPPH, 1, 1- diphenyl-1-picryl hydrazyl; DW, Dry weight; EAE, Ellagic acid equivalent; FRAP, Ferric reducing antioxidant power; GAE, Gallic acid equivalent; MS, Murashige and Skoog's medium; QE, Quercetin equivalent; RP-HPLC, Reverse phase-high performance liquid chromatography; TAE, Tannic acid equivalent; TFC, Total flavonoid content; TPC, total phenolic content; TPTZ, 2, 4, 6-tris (2-pyridyl)-s-triazine

* Corresponding author at: Department of Botany, Yashavantrao Chavan Institute of Science, Satara 415 001, India.
E-mail addresses: jaychavansu@gmail.com, jaychavan@ycis.ac.in (J.J. Chavan).

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this, shampoo ginger has wide spectrum of traditional uses as well as biological and pharmacological properties. The rhizomes of shampoo ginger has been extensively used with wide spectrum of therapeutic implications such as for the treatment of inflammation, toothache, diarrhea, indigestion, constipation, stomach cramps, bacterial infections, fever, flatulence, allergies and poisoning (Tewtrakul and Subhadhirasakul, 2007; Okamoto et al., 2011; Sidahmed et al., 2015). The crude extract and the active fractions of the rhizomes have been reported to possess antitumoral, antioxidant, anticancer, antimicrobial and antiviral properties (Murakami et al., 1999; Yob et al., 2011; Jalil et al., 2015; Koga et al., 2016).

The cone-shaped inflorescences are long-lasting and are employed in craft arrangements for ornamental purposes (Devi et al., 2014). Moreover, viscous juice present in the mature inflorescence is rich in surfactants and serves as a natural shampoo (Nalawade et al., 2003). Koga et al. (2016) reported that, shampoo ginger has great potential for cultivation that does not require high costs. Additionally, it has gained agricultural importance due to its aptitude as a putative resistance donor (resistance to soft rot causing fungus, *Pythium aphanidermatum*) to *Zingiber officinale* (Kavitha and Thomas, 2007, 2008; Nair and Thomas, 2013).

The rhizomes of many *Zingiber* species including *Z. officinale* are rich source of pungent compounds such as gingerols and shogaols (Pawar et al., 2011), which are absent in *Z. zerumbet*. However, the presence of principal bioactive compounds such as zerumbone, curcuminoids and other oleoresins confer the distinctive flavor, aroma and medicinal implications of *Z. zerumbet* (Koga et al., 2016). Zerumbone, a monocyclic sesquiterpene and important antitumoral compound is the main component of *Z. zerumbet* (Huang et al., 2005; Norulaini et al., 2009; Kitayama, 2011; Padalia et al., 2018). It has great demand in cosmeceutical and pharmaceutical industries (Murakami et al., 2002). Moreover, it was figured out that zerumbone could be used as chemopreventive agent in hepatocarcinogenesis (Taha et al., 2010). Curcuminoids; a group of bioactive polyphenolic components (curcumin, demethoxycurcumin and bisdemethoxycurcumin) are valued universally as food additives and coloring agents (Sathishkumar et al., 2015). These components chiefly present in *Z. zerumbet* (Sreevani et al., 2013; Sathishkumar et al., 2015) and have shown wide range of biological and pharmacological actions (Lopez-Lazaro, 2008).

Due to numerous health benefits, this herb deserves special attention and greater diffusion of its culture as much as *Z. officinale*. In this view, biotechnological implications especially plant tissue culture is necessary for the large-scale production of disease free, true-to the types as well as productive clones of *Z. zerumbet*. However, the genetic control over morphological as well as biochemical traits remains one of the key problem associated with commercial production of aromatic and medicinally important plant through tissue culture to supply the crude drugs of uniform and stable quality (Nayak et al., 2011). To overcome such intricacy, it is necessary to evaluate the drug yielding capability of *in vitro* raised clones and field evaluation is essential to determine the stability of drug yielding traits. All available literature dealing with plant tissue culture of shampoo ginger lacks detailed analysis of *in vitro* and subsequent field evaluation for their drug yielding potential only with single exemption except Idris et al. (2009).

The present study aims to develop an efficient, rapid and improved *in vitro* micropropagation system for shampoo ginger. We also reported the metabolic profiling, antioxidant properties and drug yielding potential across various *in vitro* conditions and subsequent field grown stages of *Z. zerumbet*.

2. Materials and methods

2.1. In vitro regeneration system

2.1.1. Plant material, sterilization and establishment of cultures

Rhizomes of mature plants of *Z. zerumbet* were collected from Kudal locality of the Northern Western Ghats of India. The rhizome was stripped off all leaves and excised into 2–3 cm in length and treated with 5% (v/v) aqueous solution of a liquid detergent 'Laboline' (Qualigen, India) for 10 min followed by thorough washing with running tap water for 30 min. The rhizome pieces containing buds were surface sterilized by immerging in 70% alcohol for 30 s followed by treatment with aqueous solution of 0.1% $HgCl_2$ for 6 min with occasional swirling and finally, washed three times with sterilized distilled water for 7 min each. Individual rhizome explants (0.4–0.7 cm) with single bud each, were excised aseptically from the sterilized rhizome pieces and transferred to culture vessels containing MS medium (Murashige and Skoog, 1962). The culture media were adjusted to pH 5.8 before autoclaving at 121 °C for 20 min. The cultures were kept at 25 ± 1 °C with 16 h photoperiod at light intensity of 36 μmol m^{-2} s^{-1} provided by cool fluorescent tubes (Philips, India).

2.1.2. Multiple shoot production, rooting and field transfer

Different concentrations and combinations of 6-benzylaminopurine (BAP), kinetin (KN), thidiazuron (TDZ) and indole-3-butyric acid (IBA) were tested for multiple shoot production as well as *in vitro* rooting. Different growth parameters such as shoot/root induction frequency (%), average number of shoot/roots and their length (cm) were recorded after 4 weeks of culture. Subsequent sub-cultures were carried out on optimal media combination for industrial-scale production of shampoo ginger. The plantlets were acclimatized in the pots containing a mixture of autoclaved soil and coco peat (1:1) and were kept in green house for 30 days. The *in vitro* raised plants as well as conventional rhizome buds were grown in black loamy soil and were watered by drip irrigation. Rhizomes of these plants were collected after 3 months interval till dormancy for chemical extraction.

2.2. Estimation of polyphenols and antioxidative activities

2.2.1. Preparation of plant extract

Fresh roots/whole plants/rhizomes were obtained from various *in vitro* conditions as well as from subsequent field cultivation stages. The rhizomes of conventionally grown plants were collected from the field. After washing under tap water, the roots and whole plant were cut into small pieces. The rhizomes were washed under running tap water and the skin was separated and the rhizomes were cut into small pieces. The small pieces of roots, whole plant as well as rhizomes were shade dried and ground into fine powder. Briefly, 0.5 g ground sample was extracted with 50 ml of methanol on an orbital shaker (Rivotek, Riviera, India) for 360 min by keeping a speed of 150 rpm at room temperature. The mixture was then centrifuged at 10,000 rpm for 10 min, filtered through Whatman filter paper No. 1 and then transferred to tubes. All extracts were stored at −20 °C prior to use in analyses. All the chemicals, standards compounds and reagents used in the study were of highest analytical grades.

2.2.2. Quantification of total phenolic content (TPC)

The quantification of TPC was carried out using modified Folin–Ciocalteu method (Wolfe et al., 2003). The absorbance of blue color developed was measured at 760 nm on double beam spectrophotometer (Thermo Scientific, multiskan Go 1510, USA). The extracts prepared were quantified and the results were compared with standard curves of tannic and gallic acid was expressed as milligram equivalent per gram dry weight.

2.2.3. Quantification of total flavonoid content (TFC)

Total flavonoid content was quantified by using aluminium chloride calorimetric method (Chang et al., 2002). The absorbance of extracts and standard solutions was measured at 367 nm. The results were expressed as milligram of quercetin and ellagic acid equivalent per gram dry weight.