Pak.J.Bot., 24(1): 54-59, 1992.

TISSUE CULTURE STUDIES FOR MICROPROPAGATION AND EXRACTION OF ESSENTIAL OILS FROM *ZINGIBER OFFICINALE* Rosc.

IHSAN ILAHI AND MUSSARAT JABEEN

Department of Botany,
University of Peshawar, Peshawar, Pakistan.

Abstract

Callus cultures of *Zingiber officinale* obtained from rhizome explants bearing shoot primordia and juve nile shoots on Murashige and Skoog basal medium supplemented with various growth hormones produced plantlets which were transferred in the field. Multiple plantlet formation occurred in two week old shoot buds. Essential oils extracted from callus cultures, from rhizomes obtained from our experimental medicinal plants garden and commercial rhizomes showed only slight difference in the oil contents.

Introduction

Rhizomes of ginger (*Zingiber officinale* Rosc.) are widely used as a medicine, spice and additive in food and beverages. Rhizomes of ginger are imported at a high cost from India and Ceylon for home consumption. Tissue culture studies were therefore initiated for micropropagation, flavor production and acclimatization of the ginger plants since tissue culture is regarded as an ideal method for producing clonal material particularly where normal propagation methods by seed or vegetative cuttings are not possible (Murashige, 1974; Holdgate, 1978).

Tissue cultures may either biotransform or produce desirable chemcials (Staba, 1977). The amount of compounds such as diosgenin (Marshall & Staba, 1977), ginseng saponins (Jhang *et al*., 1974), glycyrrhizin (Tamaki *et al*., 1973), nicotine (Shiio & Ohta, 1973), serpentine and Ajmaline (Ilahi & Akram, 1987) has approached or exceeded that in the plant. Recently cell strains containing amounts of secondary metabolites greater than those found in intact plants have been isolated by clonal selection (Yamada & Hashimoto, 1982).

Although the potential for secondary product formation in large-scale tissue culture has been recognized for some time, but little study has been undertaken for the production of food flavors. Many societies have been concerned in the use of syn thetic food, drug and beverage coloring agents and flavors, some of which are carcinogenic. New regulations governing the use of synthetic flavor and color additives to food have stimulated interest in replacing synthetic sources. Plant flavors and colors would be used more extensively if their supply was dependable and economical. Large scale tissue culture technique has therefore been suggested for providing a constant supply of compounds besides overcoming objections on toxic compounds derived from synthetic sources.

Materials and Methods

Shoot buds, rhizome explants bearing shoot primordia and juvenile shoots of ginger were thoroughly washed in running tap water then surface sterilized with 1% mercuric chloride solution for 3 minutes followed by several washings with sterilized distilled water. The explants were grown on strength 1/2 Murashige & Skoog (1962) basal medium supplemented with 5% su crose and a wide range of combinations of various growth hormones viz., IAA, NAA, 2,4-D, BA and K. pH of the medium was adjusted to 5.6 and solidified with 0.9% agar. The cultures were incubated in biotrons (incubators) with 16/8 hr light/dark cycle at 26±1°C.

Extraction of Essential Oils: The calli and the rhi zomes of *Z. officinale* were crushed and subjected to steam distillation. The steam distillate was extracted by a liquid - liquid continuous extractor for 6 h with Petroleum ether (40- 60°C). The petroleum ether solution was dried over anhydrous Na_2SO_4 which was removed by filteration. The percentage of oil in the samples was determined using the formula:

$$\% \text{ of oil} = \frac{\text{Wt. of oil obtained}}{\text{Wt. of sample}} \times 100$$

Results

Callus induction: Whereas callus was not induced in the stem explants a variety of calli were observed within the cultures of the juvenile shoots and rhizome explants bearing shoot primordia varying from whitish, soft and friable to green, hard, compact and some having segmented pouched like structures.

Combinations of 2,4-D and BA exhibited good callus growth in various explants. Callus growth was observed in the juvenile shoots on the medium supplemented with 0.5 mg/l each of BA and 2,4-D within 4 weeks of culture. Luxuriant callus growth was also obtained on a medium fortified with 0.1 mg/l of 2,4-D in combination with 0.5 mg/l of BA. The callus formed was hard and of greenish color. Copious callus formation was also observed in media containing 0.5 mg/l 2,4-D and 0.1 mg/l BA and 0.1 mg/l each of BA and 2,4-D. The tissues responded very little to the medium containing low hormonal concentrations.

Good callus was formed in 2 week old shoot buds on BM incorporated with 0.5 mg/l each of BA and 2,4- D. Copious amount of callus was also observed on the basal medium containing 0.1 mg/l 2,4-D in combination with 0.1 mg/l BA where the callus formed was hard and dark green in colour with some pouched - like structures (Fig.1). Slight rupture and small callus formation was observed on rhizome explants bearing shoot primordia on BM sup plemented with 0.5 mg/l each of BA and 2,4-D.

In vitro Plantlet Formation: Excised calli of 2 week old shoot buds, rhizome explants bearing shoot primordia and juvenile shoots were subcultured on to the basal medium supplemented with different combinations of various growth hor mones. Calli inoculated on the medium supplemented with NAA and K did not increase in size. Only a slight increase in the callus obtained from the juvenile shoots was observed on a medium fortified with 0.5 mg/l each of NAA and K. Rooting was however observed on transferring

Fig.1. Callus formation on BM supplemented with 0.1 mg/l BA and 0.1 mg/l 2,4-D.

the callus to a medium supplemented with 0.5 mg/l NAA and 0.1 mg/l K. There was no response in cultures obtained from rhizome explants bearing shoot primordia on media containing NAA and K.

Excellent callus proliferation with shoot and root formation was observed in all the cultures containing different combinations of BA and 2,4-D (Table 1). Where callus obtained from rhizome explants bearing shoot promordia were subcultured on the basal medium containing different combina tions of BA and 2,4-D, lateral buds developed on the calli in BM supplemented with 0.1 mg/l each of BA and 2,4-D and 0.1 mg/l 2,4-D with 0.5 mg/l BA. Some bud formation was also observed on a medium supplemented with 0.5 mg/l each of BA and 2,4-D.

Excised callus portions developed from juvenile shoots were subcultured on different combinations of BA and 2,4-D. Excellent callus growth was observed on the medium supplemented with 0.1 mg/l of 2,4-D in combination with 0.5 mg/l BA. Moreover small green spots were noticed in these cultures after 3rd week of subculture, which ultimately grew into small plantlets (Fig.2). An average of 30 plantlets were obtained per g of callus in about 4 weeks.

To obtain a high frequency of regeneration, K was used as an additional supplement in combination with 2,4-D and BA in the medium. Luxuriant callus growth were found on most of the kinetin supplemented media. However, addition of K did not have an effect on bud initiation. The callus increased in bulk in most of the concentrations of K used and greening was also observed in the medium with no bud initiation.

Percentage of Volatile Oils: Percentage of oil obtained from callus culture was 0.159 whereas that obtained from ginger rhizomes was 0.169. The color and odor of oils ob tained from both the samples was similar.

Discussion

The results would suggest that callus induced in various explants could be successfully and continuously subcultured. Moreover, organogenesis i.e., root and shoot and

Table 1. The influence of BA and 2,4-D on callus growth in ginger.

Explant	Hormone concentration mg/l Previous	Present	Callogenesis	Morphogenesis	Culture period in weeks
Two weeks old sprouting buds	0.5 2,4-D +0.5 BA		+ + +	-	4
"	0.5 2,4-D +0.5 BA	1/2 MS	-	Roots	4
"	0.5 2,4-D +0.5 BA	0.05 2,4-D +0.05 BA	-	Root formation occurred	4
"	0.5 2,4-D +0.5 BA	0.1 2.4-D +0.1 BA	+ + + + green Pouched callus	Root hairs	4
"	0.5 2,4-D +0.5 BA	0.5 2,4-D +0.5 BA	+ + + + friable callus	Roots formed	4
"	0.5 2,4-D +0.5 BA	0.1 2,4-D +0.5 BA	+ + + +	-	4
	0.5 2,4-D +0.5 BA	0.5 2,4-D +0.1 BA	+ + + + compact callus	-	4
Rhizome explant bearing shoot primordia	0.5 2,4-D +0.5 BA	-	+	-	4
"	0.5 2,4-D +0.5 BA	0.1 2,4-D +0.5 BA	+	Lateral buds with multiple shoots	4
"	0.5 2,4-D +0.5 BA	0.5 2,4-D +0.1 BA	+	Lateral buds with multiple shoots	4
"	0.5 2,4-D +0.5 BA	0.1 2,4-D +0.1 BA	+	"	4
"	0.5 2,4-D +0.5 BA	0.5 2,4-D +0.5 BA	+	lesser no. of shoots	4
Juvenile shoot	0.5 2,4-D +0.5 BA	-	+ + + Callus		
"	0.5 2,4-D +0.5 BA	0.5 2,4-D +0.5 BA	+ + + + with green region	shoots buds turning into plantlets	6
"	0.5 2,4-D +0.5 BA	0.5 2,4-D +0.1 BA	+ + +	Shoot buds & roots	4
"	0.5 2,4-D +0.5 BA	1.0 2,4-D +0.1 BA	+ + +	Roots	4
"	0.5 2,4-D +0.5 BA	0.5 2,4-D 0.5 BA	+ + + + whitish green with dark green nodulated areas	Plantlets	4
"	0.5 2,4-D +0.5 BA	0.1 2,4-D +0.1 BA	+ + +	Plantlet formation with larger no. of roots	4

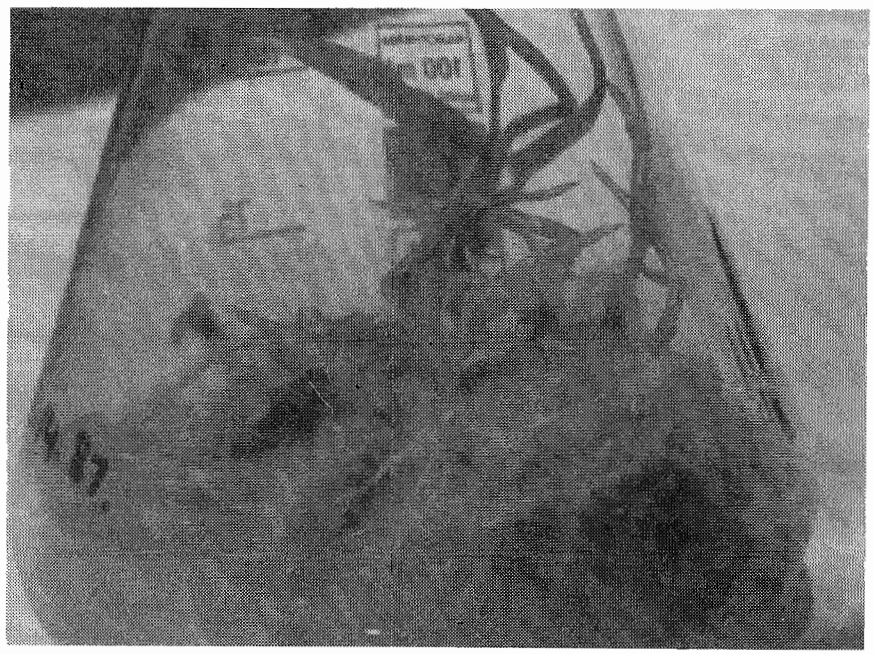

Fig.2. Plantlet formation on BM supplemented with 0.5 mg/l BA in combination with 0.1 mg/l 2,4-D.

complete plantlets with the formation of daughter buds was obtained on further subculturing of the induced callus. Success in the technology and application of *in vitro* methods as a means of plant propagation is due largely to a better understanding of the nutritional requirements of the cultured cells and tissues (Gamborg *et al.,* 1976; Murashige, 1974; Street, 1977).

In the present studies, callus could not be induced on stem explants even after using a wide range of different hormonal concentrations. Tissues from very many organs may have an ulti mate potential to divide and proliferate when transferred to culture medium. In most plants, tissues from some organs are more predisposed to rapid cell division than others. It is this difference which is particularly apparent in monocotyledons. In most cereals, e.g., callus growth can only be obtained from tissues of certain organs such as zygotic embryos, germinating seeds, seed endosperm or the seedling mesocotyl, but so far never from mature leaf tissue (Green & Phillips, 1975).

Of the various growth hormones used, combinations of 2,4-D and BA proved to be the best both for callus formation and morphogenesis. Calli cultured on medium containing a combination of NAA and kinetin did not increase much in size nor was any shoot development observed in any of the cultures, however root formation was observed in nearly all of the cultures. These results are in contrast to those obtained by Akram (1983). He reported shoot regeneration in callus cultures of *Rauwolfia serpentina* on a medium supplemented with a combination of NAA and a cytokinin whereas root initiation was very slow in such combination.

The calli tested at different stages showed that flavors of ginger could be found only in dark green organogenic type of calli which already differentiated into roots and shoots or was near to differentiation. Soft and friable calli were tasteless. Oil could also be obtained only from organogenic calli. Similarly Becker (1970) found no essential oil synthesis in tissue culture of a variety of plant herbs until redifferentiation. Fridborg (1971), Freeman *et al.*, (1974) and Turnbull *et al.*, (1981) also demonstrated that redifferentiation of onion tissue cultures into roots or shoots was essential for the pro duction of onion flavour. In cellery cultures embryogenesis was essential before the cellery flavour was formed (Al-Abta *et al.*, 1979).

Acknowledgements

This study was supported by National Science Foundation, U.S. under grant No. INT-7826889 to I.I. The financial support is gratefully acknowledged.

References

Akram, M. 1983. *Studies on Tissue Culture of Rauwolfia serpentina* (L) Benth Ex Kurz. Ph.D. Thesis, Department of Botany, University of Peshawar, Peshawar, Pakistan.

Al-Abta, S., I.J. Gapin and H.A. Collin. 1979. Flavor compounds in tissue cultures of celery. *Plant Sci. Lett.*, 16: 129-134.

Becker, H. 1970. Untersuchungen zur Frage der Bildung fluechtiger Soff wechset produckte in callus kultunen. *Biochem. Physiol. Pflanz.*, 161: 425-441.

Freeman, G.G., R.J. Whehham, I.A. Mackenzie and M.R. Davey, 1974. Flavour components in tissue cultures of onion (*Allium cepa* L.). *Plant Sci. Lett.*, 3: 121-125.

Fridborg, G. 1971. Growth and organogenesis in tissue cultures of *Allium cepa* v. proliferum. *Physiol. Plant.*, 25: 436-440.

Gamborg, O.L., T. Murashige, T.A. Thorpe and I.K. Vasil. 1976. *In vitro.*, 12: 473.

Green, C.E. and R.L. Phillips. 1975. Plant regeneration from tissue cultures of maize. *Crop Sci.*, 15: 417-421.

Ilahi, I and M. Akram. 1987. Leaf callus culture of *Rauwolfia serpentina. Pak.J. Bot.*, 19: 217-223.

Jhang, J.J., E.J. Staba and J.Y. Kim. 1974. American and Korean ginseng tissue cul tures: growth, chemical analysis and plantlet production. *In vitro*, 9: 253-259.

Marshall, J.G. and E.J. Staba. 1976. Hormonal effects on Diosgenin hiosynthosis and growth in *Dioscorea deltoidea* tissue cultures. *Phytochemistry*, 15: 53-55.

Murashige, T. 1974. Plant propagation through tissue culture. *An. Rev. Plant Physiol.*, 25: 135-165.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.

Shiio, I., and S. Ohta. 1973. Nicotine production by tobacco callus tissues and effect of plant growth regulators. *Agr. Biol. Chem.* 37: 1857-1864.

Street, H.E. 1977. In: *Plant Tissue and Cell Culture*. Botanical Monographs. Vol.II. 2nd Edition, Balckwell. Oxford.

Tamaki, E., I. Morishita, K. Nishida, K. Kato, T. Matsumoto. 1973. Process for pre paring licorice extract like material for tobacco flavoring. U.S. Patent No.3, 710, 512.

Turnbull, A., I.J. Galpin, J.L. Smith and H.A. Collin. 1981. Comparison of the onion plant (*Allium cepa*) and onion tissue culture. IV. Effect of shoot and root morphogenesis of flavour precursor synthesis in onion tissue. *New Phytol.*, 87: 257- 268.

Yamada, Y. and T. Hashimoto. 1982. Production of Torpane alkaloids in cultured cells of *Hyoscymus niger*. *Plant Cell. Rep.*, 1: 101-103.

(Received for Publication 21 November 1990)