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Journal of Essential Oil Bearing Plants

ISSN: 0972-060X (Print) 0976-5026 (Online) Journal homepage: http://www.tandfonline.com/loi/teop20

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To cite this article: Sapna Baghel & Yogendra Kumar Bansal (2015) In vitro Regeneration of Oil Yielding Plants-A Review, Journal of Essential Oil Bearing Plants, 18:5, 1022-1050, DOI: 10.1080/0972060X.2014.971068

To link to this article: http://dx.doi.org/10.1080/0972060X.2014.971068

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ISSN Print: 0972-060X
ISSN Online: 0976-5026

In vitro Regeneration of Oil Yielding Plants-A Review

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Received 10 June 2014; accepted in revised form 25 November 2014

Abstract: Micropropagation of different oil yielding plants has played a very important role in rapid multiplication of cultivars with desirable traits and production of healthy and disease-free plants with good oil yield. During the last several years, different strategies have been carried out for *in vitro* propagation of different oil yielding plants. The objective of this review is depiction of different plants that producing biodiesel to fulfil the demand of fossil fuel. The plants reported are promising biofuel and bioenergy crops possessing high biomass and oil quality. Micropropagation through apical and axillary shoot proliferation, while adventitious shoot proliferation from leaf, internode and zygotic embryos explants, or both, has been successful and influenced by several internal and external factors viz. Genotypes/ Cultivars, Media, Carbohydrates, Form of medium, Growth regulators, Light, Temperature, Relative humidity specific need during stages of micropropagation like establishment of *in vitro* cultures, shoot multiplication, rooting of *in vitro* regenerated shoots and acclimatization are discussed in the present review. On the whole rapid regeneration and multiplication through organogenesis or somatic embryogenesis is discussed in this review.

Key words: *In vitro* regeneration, Shoot multiplication, Plant growth regulators, Rooting, Biodiesel.

Introduction

Fossil fuels have always been the principal source of energy for steering infrastructural and economic development both in the developing as well as developed countries [1,2]. However, since fossil fuels provide limited source of energy for alternative sources of energy are required that would be economically efficient, socially equitable and environmentally sound.

The term ‘Biodiesel’ was introduced in the United State during 1992 by the National Soy Diesel Development Board (Presently known as National Biodiesel Board) which has already pioneered the commercialization of biodiesel in United States. The American Society for Testing and Materials (ASTM) defines biodiesel fuel as “monoalkyl esters of long chain fatty acids derived from a renewable lipid feedstock, such as vegetable oil or animal fat”. ‘‘Bio’’ represents its renewable and biological source in contrast to traditional petroleum-based diesel fuel and ‘‘diesel’’ refers to its use in diesel engine.

Importance of Biodiesel

Alternative fuel, energy conservation management, energy efficiency and environmental protection have, therefore, become critically important in recent years [3]. The increasing import bill necessitates the research of liquid fuel as an alternative to diesel, which is being used in large quantities in transport, agricultural, industrial, commercial and domestic sectors. Therefore, biodiesel obtained from vegetable oils has been considered as a promising option [4].

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Production of biodiesel from plant oil

The world market for biodiesel has expanded rapidly in recent years. A large number of countries have already implemented a broad range of laws that support the usage of biodiesel. At present, a biodiesel mandate for use as motor fuel has been set in various countries with various incentives and support. Typical raw materials for biodiesel are mainly Rapeseed or Sunflower oil in Europe, USA as well as Canada uses Soybean, Rapeseed, other waste oils and fats. Frying oil and animal fat has been kept as an option in Ireland; Castor oil and Soybean oil are used in Brazil; Coconut oil and Palm oil are preferred in Malaysia, Philippines, Thailand and Indonesia. Cotton Seed oil in Greece; Linseed and Olive oil in Spain; Jatropha and Karanja are used in India, Nicaragua as well as Africa to produce biodiesel [5-7]. Several other non-edible plants such as Neem (*Azadirachta indica*), Thumba (*Citrullus colocynthis*), Indian butter tree (*Diploknema butvracea*), Saptree (*Garcinia* Species), Rubber (*Hevea* species), Mahua (*Madhuca indica*), Castor (*Ricinus communis*) and Meswak (*Salvadora* species) may also be used for producing biofuels in India [8-10].

The suitability of vegetable oil for a particular use viz. nutritional, industrial or pharmaceutical is determined by its fatty acid composition which is highly variable depending on the plant species. This has encouraged researchers to look for new sources of oil or a new fatty acid composition in different plant species. Genetic variation for fatty acid composition is essential for genetic improvement of the oil quality and developing new cultivars. A large number of potential plants have been identified and analysed for oil content and fatty acid profiles and are cultivated as the new oil seeds crop [11].

Problems of conventional breeding

In order to increase yield and seed quality of oil yielding plants, it is necessary to agronomically improve its important traits, such as herbicide resistance, disease resistance, tolerance to several biotic, abiotic stress factors and fatty acid compositions [12]. Improvement of plants through conventional breeding methods is slow, time-consuming and labour-intensive. Modern genetic improvement techniques which are based on molecular genetics and tissue culture have, therefore been replacing the conventional breeding methods [13]. Commercial production of plants through micropropagation techniques has several advantages over the traditional methods as it can lead to the production of virus free plants [14].

Importance of plant tissue culture

It is a technique for the multiplication of plants by which any plant parts can be cultured on a nutrient medium under sterile conditions with the purpose of obtaining growth. The term "*in vitro*" refers to "in glass" or in an artificial environment compared to "*in vivo*" which means "in soil" [15]. Tissue culture technique is a powerful tool which can be employed as an alternative to the conventional method of vegetative propagation with the objective of enhancing the rate of multiplication of desired genotypes [16,17].

In vitro regeneration in plant tissue culture occurs through two main pathways, either directly from the explants (direct organogenesis) or through a callus phase (indirect organogenesis). *In vitro* plant regeneration via organogenesis is controlled primarily by the interaction of plant hormones, specifically cytokinins and auxins with plant tissue in culture medium [18]. Biotechnological tools like Organogenesis, Somatic embryogenesis, Synthetic seed, Suspension culture, Protoplast culture, Haploid culture and Molecular markers offer a valuable alternative to plant diversity studies, management of genetic resources and eventually result in their conservation [19].

Plant tissue culture technology has been valuable to the plant breeders for nearly four decades and has been extensively employed for crop improvement of several oil crops [20]. The tissue culture technique is used for propagation, genotype modification as well as biomass production of germplasm.

Micropropagation of oil yielding plants

Stages involved in micropropagation

A successful micropropagation protocol proceeds through a series of stages, each with a specific set of requirements. These are (i) initiation of aseptic cultures, (ii) shoot multiplication, (iii) rooting of *in vitro* regenerated shoots and (iv) hardening and field transfer of tissue culture raised plants.

Initiation of aseptic cultures

Choice of explants

The choice of explant for initiation of culture is largely dictated by the method to be adopted for *in vitro* propagation. Rapid clonal plant propagation *in vitro* can be obtained through bud or shoot proliferation. A single explant source, embryo, shoot tip, hypocotyl, leaf, nodal segment could conceivably provide thousands of new "true to type" plantlets per year. The difference in responses of the explants types are probably due to endogenous hormonal balance in plant tissues [21]. Although different researchers have used different explants for regeneration, the most commonly used explants are apical and nodal stem segments, wherein the axillary bud is made to proliferate to form multiple shoots [22-27] (Table 1) while zygotic embryo is most commonly used for callus induction [28-30] (Table 1) and somatic embryo formation [31-36] (Table 1).

Disinfection and surface sterilization of explants

For *in vitro* culture initiation, seeds or explants (directly from plants) are normally collected from field grown plants, so the plant material is liable to be contaminated by microorganisms which must get disinfected before explants are transferred to *in vitro* conditions. Variation in sterilization procedures have been proposed by many researchers. Several surfactant or disinfectants such as Labolene (Teepol) [20, 37-41], Savalon [32,42], Benlate [43], TRITON-X-100® [44], Decon 90 [34], Calcium hypochlorite [25,45], Domestos [46,47], PPM™ [47], Mercuric chloride [33,35,41,48-51], Axion [52], Chloroxylenol, Sodium hypochlorite (active chlorine) [33,36,53-58], Hydrogen peroxide [59], Silver nitrate [60], Tween 20 [36,55,61,62], Tween® 80 [63,64], Detertec (Vetec, Brazil) [33], Clorax [23,26,65] and Ethyl alcohol [36,55,65-68] etc. have been used for the surface decontamination of variety of explants. Different antibiotics (gentamycin, ampicillin, tetracycline or amoxicillin) [60] and Bavistin® (antifungal agent) [69-72] at different concentrations and duration for disinfection from internal contaminants have also been used and subjected to repeated washings in sterile distilled water.

Browning of the medium

The medium in which explants are grown become coloured within an hour or two after planting the material as observed in many tropical and sub-tropical woody species [73]. The brown and black colour development in culture is due to the formation of quinines possibly as a result of binding between phenol and proteins and its subsequent oxidation to quinines a loss of enzyme activity might result [74,75]. To prevent the leaching of polyphenols from the cut ends of the explants various antioxidants are incorporated in the medium such as activated charcoal, ascorbic acid, citric acid [60], DIECA (sodium diethyl thio-carbamate) [46] and PVP (polyvinyl pyrrolidone). Incubation of explants in darkness prior to inoculation also helps in reducing the browning problem by preventing or reducing the activity of enzyme concerned with both biosynthesis and oxidation of phenols [76]. Regular subculturing of explants on fresh medium is another simple and successful method to protect plants from the detrimental effect of oxidative browning [22].

Shoot multiplication

This is the most crucial stage of micro-propagation. The success of a micropropagation protocol depends on the rate and mode of shoot multiplication to a large extent. Various factors that influence *in vitro* shoot multiplication in oil yielding plants are listed below.

Species/genotypes/cultivars

It is well known that *in vitro* culture is dependent on the genotype of donor material. In fact different type of morphogenic responses eg. somatic embryogenesis, organogenesis, shoot proliferation and rooting *in vitro* are strongly determined by the genotype of the explants [77]. This probably indicates that specific genotypes possessing specific genetic combinations are more likely to undergo a particular type of morphogenesis than others [78]. One of the researchers [79] has marked a clear effect of genotypes on *in vitro* propagation in *Brassica juncea.* He observed that *B. juncea* AB79/1 (genotype 2) and *B. juncea* I39/1 (genotype 1) showed a greater capacity to produce shoots than *B. juncea* J99 (genotype3). Other researchers [48] have also reported in *Beta vulgaris* that breeding line Line ELK345 gave high number of shooting than Line M114 and Line M1017. In *Avena sativa* 10 oat genotypes (Ankara-76, Ankara D 84, A- 803, A- 804, A- 805, A- 821, A-822, A- 823, A- 824 and A- 825) have been used showing maximum regeneration capacity in A-824 [29]. Some author has reported in their study that out of four cultivars of *Brassica napus* used to evaluate shoot regeneration viz. Jumbo, Drakkar, Cossair and Pactol. The former two showed a greater capacity to produce shoots on the medium [45]. In *Cucurbita pepo* out of two cultivars viz. Bulum and Rumbo used, better organogenesis was observed in cv. Bulum [80]. In *Gossypium hirsutum* five genotypes DCH-32, DHY-286, LRA-5166, LRK-516 and AKH-081were tested for shoot regeneration and multiplication but best response was observed in LRA-5166 [41]. In *Helianthus annuus* effect of genotype variation was studied, highest mean shoot number was obtained in cv. Hysun 45 to none at all in cv. DL 9542 [81]. Some researcher achieved high percentage of regeneration and multiplication in Aglandau cultivar than Tanche and Laragne of *Olea europaea* [81]. Some author noticed that the maximum number of callus were regenerated in SPTG-172 whereas the mean number of shoots per callus at all concentration was higher in K-399 indicating that shoot regeneration is markedly affected by the genotype of *Nicotiana tabacum* [66]. In *Melia azedarach* all six clones (3, E, H, J2, Lp and 20) has produced multiple shoots and E regenerated the greatest mean number of shoots per explant [44]. Some author reported that among the 3 genotypes, the explant of cultivars 'Szaphir' of *Linum usitatissimum* produced the best results as maximum percentage of regeneration frequency and number of shoots than 'MIkael' and 'Barbara' cultivars [55]. In *Sorghum bicolor*, it was found that out of 2 genotypes (K8 and K5) used K8 was more favourable for shoot regeneration and multiplication. Therefore, K8 genotype was selected for further study [20]. some author studied on *Sesamum indicum*. They found that out of different cultivars viz. Busia, Ex-El, Koyonzo, Mbale, McWhite, Mtwara-2, Siaya and one Indian cultivar. The best cultivar Ex-El scored the highest regeneration frequency and multiplication of shoots per explant while the lowest regeneration frequency and number of shoots produced per explant were recorded for Siaya and McWhite respectively [53]. However, in *Carya illinoinensis* both 'Cape Fear' and 'Desirable' cultivars no differences were observed in terms of regeneration capacity and shoot multiplication [56].

Medium

Plant tissue culture media generally contains some or all of the following components: macronutrients, vitamins, amino acids or nitrogen supplements, source(s) of carbon, undefined organic supplements, growth regulators and solidifying agents.

Selection of an appropriate culture medium and the use of correct growth regulators are critical for the optimum growth response of the explants. Different type of media have been attempted on various oil yielding plants such as WPM [82] was used by many researchers [25,56,63,69,83,84], B_5 medium [85] were used by some researchers [86,87]. DKW[88] or Eeuwen's medium or Y3 medium [89] were used by some researchers [24,34,90-92]. Many researchers [29,47,60,93] have also used MS [94] medium and Chee and Pool (C_2D) vitis [95] medium [27]. The most widely used culture medium is MS medium, because most of the plants respond to it favourably. It is classified as a high salt medium as compared to other formulations, with high levels of nitrogen, potassium and some of the micronutrients, particularly boron and manganese[96]. Other researchers also modified the MS medium to improve response and regeneration potential [46].

Carbohydrate

Carbohydrate acts as a source of energy and as osmotic agent as well [97]. Sucrose (3 %) is supplied as the main carbohydrate in the medium, different concentrations of which were studied by different researchers [47, 65, 98]. However, there are many examples on the use of higher concentrations of sucrose both for shoot initiation and proliferation [55, 91, 92]. Sometimes, sucrose may compensate for the lack of PGRs in culture medium for shoot regeneration [45]. The use of fructose and glucose in the culture medium for better shoot proliferation has also been reported in *Corylus avellana* [89] and *Zea mays* [39].

Form of medium

Interaction between *in vitro* raised plantlets with the gelling agent in culture medium is a dynamic process and the changes in gel consistency affect the regeneration of plants or tissues [99]. In tissue culture work, different gelling agents have been used for solidifying the culture medium. Agar is the most commonly used gelling agent because of its desirable characteristics such as clarity, stability and inertness [100]. It is a complex poly-saccharide obtained from some species of algae. During fabrication it is subjected to the variation in degree of purification. However, mineral and organic impurities are retained by it [101]. The most popular alternative to agar is Phytagel or Gelrite (Gellan gum) which is a complex extra-cellular polysaccharide produced by *Pseudomonas elodea*. Gelrite contains less free minerals and impurities than agar [102]. In contrast to the medium solidified agar, pH often drops as the culture ages. Whereas in medium solidified with Gelrite, pH tends to be more stable [103]. Generally 0.6-0.8 % of agar is used as a gelling agent; however, 0.2-0.4 % of phytagel has been used in some cases [24,33,34,54,56,65,87,104]. *In vitro* propagation in liquid medium has also been attempted where agar was completely omitted from the medium. By using liquid medium instead of gelled medium, propagation is accelerated in *Carya illinoinensis* [56] and *Miscanthus giganteus* [105]. This increased availability may be induced by a lower resistance to diffusion and closer contact between the explant and the culture medium.

Plant growth regulator

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators provided in the medium and those produced endogenously by an explanted tissue, while most growth regulators exert a direct effect on endogenous growth substances [106]. Moreover, the formation of adventitious organs depends on the reactivation of genes concerned with the organogenic phase of development.

It is well known that cytokinin stimulate plant cell division and participate in the release of lateral bud dormancy, induction of adventitious bud formation, growth of lateral buds and in cell cycle control [107]. Cytokinins have been reported to play a key role in DNA synthesis and cell division, which might be the reason for induction of multiple shoots [108] Inclusion of cytokinins viz. BAP, TDZ, KN, ZT and m-Topolin in the culture medium has been found essential for bud break and shoot multiplication in various plants like *Aegle marmelos* [41], *Calophyllum apetalum* [22], *C. inophyllum* [69], *Cannabis sativa* [70], *Carya illinoinensis* [56], *Ceiba pentandra* [109], *Cleome viscosa* [59], *Cucurbita pepo* [80], *Euphorbia lathyris* [110], *Guizotia abyssinica* [111], *Olea europaea* [25], *Panicum virgatum* [112], *Pongamia pinnata* [113], *Psophocarpus tetragonolobus* [51], *Raphanus sativus* [114], *Sclerocarya birrea* [57], *Sterculia foetida* [48], *Terminalia bellerica* [115], *Theobroma cacao* [24] and in *Ziziphus spinachristi* [23] (Table 1).

To encourage the growth of explants in shoot cultures, one or more cytokinins are usually incorporated into the medium at initial establish-ment stage [106]. Some authors have used BAP in combination with KN for shoot initiation and multiplication in *Annona squamosa*, *Cerbera odollam* and *Terminalia catappa* [26, 60, 116] (Table 1). However, in *Hibiscus sabdariffa* BAP in combination with m-Topolin has been used for shoot multiplication [54] (Table 1). In some cases, BAP in combination with adenine produce multiple shoots in *Simmondsia chinensis* [50] (Table 1). The benefits of using a combination of cytokinins rather than a single compound may be an indication of differences in uptake, recognition by the cells or mechanism of action of the compounds [117].

In another study, some researchers [84, 118] reported that BAP in combination with GA_3 also results in shoot initiation as well as multiple shooting in *Tectona grandis* and *Passiflora edulis* respectively

Table 1. Few reports on the tissue culture studies on multipurpose oil yielding plants used for biodiesel production

Species	Habit	Explant used	Medium used	PGRs (mgl^{-1}) and other supplements	Result obtained	Reference
Acrocomia aculeata	Tree	Zygotic embryos	MS	Y3 salts+Hydrolizedcasein+ Myo-inositol+Picloram	Calli induction	33
				AC	Somatic embryo formation	
Aegle marmelos	Tree	Nodal Segments	MS	BAP	Shoot multiplication	41
				BAP+ KN+ GA_3	Shoot elongation	
			½ MS	IBA + AC	Rooting	
Aleurites moluccana	Tree	Stem	½MS	ZT+NAA+choline chloride	Callus formation	151
				BAP+ IBA	Axillary bud formation	
Anacardium occidentale	Tree	Nucellar tissue	MS	2-4D+ GA_3+ BAP	Callus formation	98
				2-4D+ GA_3+ CW+ CH+ AC	Somatic embryo formation	
Annona squamosa	Tree	Nodal segments	MS	BAP+ KN	Shoot initiation	60
				KN+ AC	Shoot elongation	
			White	IBA+ AC	Rooting	
Arachis hypogaea	Legume	Cotyledonary node	MS	Picloram	Somatic embryo initiation	65
				B_5 vitamins +2-4D+NAA	Callusing	
				BAP+IAA	Regeneration+ Elongation	
				NAA	Rooting	
Arundo donax	Shrub	Axillary buds	MS	2,4-D+ BAP	Callus induction	93
				-	Plant regeneration	
Avena sativa	Cereal grain	Mature embryo	MS	2,4-D	Callus inductiom	29
				-	Shoot and Root formation	
Azadirachta indica	Tree	Root	MS	BAP+ 2iP+ IAA+ adenine hemisulphate+ putrescine	Shoot initiation and multiplication	136
			IBA	Rooting		
Balanites aegyptiaca	Tree	Shoot tips	C_2D	KN+ NAA+ $AdSO_4$	Multiple shoot formation	27
		Nodal explants		$AdSO_4$	Elongation of shoots	
				IAA+AC	Rooting	

table 1. (continued).

Species	Habit	Explant used	Medium used	PGRs (mgl^{-1}) and other supplements	Result obtained	Reference
Basella rubra	Herb	Stem, Leaf	MS	BAP+2,4-D	Callusing	154
Beta vulgaris	Herb	Petiole	MS	TDZ	Germination	47
				BAP +NAA	Multiple shoot formation	
				NAA	Rooting	
Brassica juncea	Herb	Hypocotyl, Petiole	MS	BAP+NAA +$AgNO_3$	Multiple shoot formation	79
		transverse thin cell layer		-	Rooting	
B. napus	Herb	Hypocotyl, Petiole	MS	BAP+NAA	Multiple shooting, rooting	45
Broussonetia papyrifera	Tree	Lateral bud	MS	BAP+ NAA	Multiple shoot formation	122
			IBA	Rooting		
Calendula officinalis	Herb	Hypocotyl, Cotyledon	MS	TDZ, TDZ+ IBA	Multiple shoot formation	52
				NAA	Rooting	
Calophyllum apetalum	Tree	Shoot tip,	MS	BAP	Multiple shooting	22
		Nodal segment	½ MS	-	Elongation	
			MS	IBA	Rooting	
C. inophyllum	Tree	Seedling	WPM	-	Germination	69
				TDZ	Shoot multiplication	
				BAP+IBA	Rooting	
Camelina sativa	Herb	Leaf	MS	BAP+ NAA	Multiple shooting	123
Cannabis sativa	Herb	Apical bud	MS	TDZ	Multiple shoot formation	70
			I	BA+NAA	Rooting	
Carthamus tinctorius	Herb	Root, Hypocotyl,	MS	TDZ+ NAA	Multiple shooting	134
		Cotyledon and		KN	Shoot elongation	
		Primary leaf	½ MS	NAA	Rooting	
Carya illinoinensis	Tree	Nodal explants	WPM	BAP	Multiple shoot formation	56
				-	Elongation	
				IBA	Rooting	
Caryocar brasiliense	Tree	Leaf	WPM	NAA+ BAP+ CW+ CH+ malt extract	Callusing	83

table 1. (continued).

Species	Habit	Explant used	Medium used	PGRs (mgl^{-1}) and other supplements	Result obtained	Reference
Ceiba pentandra	Tree	Apical bud	MS	BAP	Multiple shooting	109
Celastrus paniculatus	Tree	Nodal explants	MS	BAP+ NAA	Multiple shooting	124
				Exvitro rooting		
Cleome viscosa	Herb	Leaf	MS	IAA	Callusing	59
				BAP	Multiple shooting	
				NAA	Rooting	
Cerbera odollam	Tree	Shoot tip, Axillary bud	MS	BAP+ KN	Multiple shooting	26
				IBA	Rooting	
Citrullus colocynthis	Herb	Shoot tip	MS	BAP+ NAA	Shoot multiplication and elongation	125
			IBA+ AC	Rooting		
Cocos nucifera	Tree	Plumule	Y3	BAP+ 2,4-D+ AC	Callusing	91
				2,4-D+ABA	Somatic embryogenesis	
Coffea arabica	Tree	Leaf, Stem segments	½ MS	BAP +IAA + triacontanol	Somatic embryogenesis	150
Coriandrum sativum	Herb	Hypocotyl, Cotyledon	MS	2,4-D	Somatic embryogenesis	152
			½ MS	-	Plantlets	
Corylus avellana	Tree	Shoot bud	MS, DKW	BAP+ Polyamine	Shoot multiplication and elongation	90
Crambe abyssinica	Herb	Cotyledon, Hypocotyl	MS	NAA, 2.4-D	Callus formation	149
				TDZ+ BAP+ IBA	Somatic embryogenesis	
Cucurbita foetidissima	Herb	Shoot tip	MS	BAP+ IAA	Mulitiple shoot formation	132
				IBA+IAA	Rooting	
C. pepo	Herb	Hypocotyl, Cotyledon	MS	2,4-D	Callusing	80
				TDZ	Shoot multiplication	
				IBA	Rooting	
Cynara cardunculus	Herb	Embryo	MS	KN+ NAA	Multiple shoot formation	104

table 1. (continued).

Species	Habit	Explant used	Medium used	PGRs (mgl^{-1}) and other supplements	Result obtained	Reference
Elaeis guineensis	Tree	Zygotic embryo (IZEs)	Y3	-	Callusing and Somatic embryo formation	34
Eruca sativa	Herb	Cotyledonary nodes	MS	BAP+ IAA	Multiple shoot formation	62
			½ MS	IBA+ KN	Rooting, *In vitro* flowering	
Euphorbia helioscopia	Herb	Mature leaf discs	MS	2,4-D	Callusing	61
E. lathyris	Herb	Apical shoots	MS	BAP	Multiple shoot formation	110
				NAA	Rooting	
E. tirucalli	Shrub or small tree	Stem segments	½ MS	NAA + BAP +Ad	Multiple shoot formation	129
				NAA+IBA	Rooting	
Garcinia indica	Tree	Immature seeds	WPM	NAA+BAP	Somatic embryo initiation	32
				BAP/KN+IBA	Somatic embryo maturation	
Glycine max	Herb	Immature shoot tips embryonic	MS	2,4-D+ asparagines+glutamine	Somatic embryo initiation	36
Gossypium hirsutum	Shrub	Cotyledonary node	MS	BAP+NAA	Multiplication and elongation of shoots	42
Guizotia abyssinica	Herb	Leaf, Internode	MS	BAP	Multiple shoot formation	111
				BAP+ GA_3	*In vitro* flowering	
				-	Rooting	
Helianthus annuus	Herb	Cotyledon	MS	BA+ NAA	Callusing + Somatic embryo formation	81
Hevea brasiliensis	Tree	Seeds	MS	BAP+NAA	Shoot formation with roots	126
Hibiscus sabdariffa	Subshrub	Shoot apex	MS	BAP+ m-Topolin	Multiple shoot formation	54
Hordeum vulgare	Gramnoid	Mature embryo	MS	2,4-D	Callusing	30
				-	Plant regeneration	
Jatropha curcas	Shrub	Node, Leaf	MS	BAP+ IBA+ $AdSO_4$+ Glutamine+ Proline	Multiple shoot formation	40
				IBA+NAA	Rooting	
Kosteletzkya virginica	Sub shrub	Mature embryos	MS	IAA+ KN	Mature embryo formation a nd plant regenartion	148

table 1. (continued).

Species	Habit	Explant used	Medium used	PGRs (mgl^{-1}) and other supplements	Result obtained	Reference
`*Linum usitatissimum*	Herb	Hypocotyl	MS	TDZ+NAA	Multiple shoot formation	55
Lupinus mutabilis	Herb	Hypocotyl TCL Modified	MS	IAA+ BAP	Multiple shoot formation	46
Macadamia integrifolia	Tree	Nodal segments	WPM	BAP+ IBA + GA_3	Multiple shoot formation	63
Madhuca longifolia	Tree	Apical, Axillary meristems	MS ½ MS	BAP+ NAA IBA	Multiple shoot formation Rooting	140
Melia azedarach	Tree	Apical meristem	MS	BAP+IBA IBA	Multiple shoot formation Rooting	44
Michelia champaca	Tree, Shrub	Immature seeds	MS	NAA -	Embryogenic callus then somatic embryo initiation Plant regeneration	43
Miscanthus giganteus	Graminoids	Immature inflor-escence explants	MS	2,4-D+BAP BAP+IAA+IBA BAP+2,4-D+AC	Embryogenic callusing Multiple shooting Rooting	105
Momordica dioica	Climber	Nodal segments	MS ½ MS	BAP+ IAA IBA +AC	Multiple shoot formation *ex vitro* rooting	67
Moringa oleifera	Tree	Nodal	MS IAA+IBA	BAP+ Triacontanol+ NAA Rooting	Multiple shoot formation	58
Myristica malabarica	Tree	zygotic embryos	MS	2-iP+TDZ+ AC	Somatic embryo formation	35
Nicotiana tabacum	Herb	Leaf	MS	BAP+ NAA	Callusing then shoot formation	66
Olea europaea	Tree	Nodal segments, ApB	OM/WPM	ZT IBA/NAA	Multiple shoot formation Rooting	2
Oryza sativa	Graminoids	Seeds	MS	2,4-D+Proline + KN BAP+KN+NAA	Embryogenis Callus initiation Shoot formation	71
Panicum virgatum	Graminoids	Inflorescence	B_5	BAP	Callusing, Shoot initiation	112
Papaver somniferum	Herb	Hypocotyl	MS	KN+ NAA 2,4-D+NAA	Callusing Somatic embryo formation	153

table 1. (continued).

Species	Habit	Explant used	Medium used	PGRs (mgl^{-1}) and other supplements	Result obtained	Reference
Passiflora edulis		Shoot tip	MS	BAP+GA_3	Multiple shooting	118
Persea americana	Tree	Mature and j uvenile Stem	MS	Peptone+ BAP+2,4-D Peptone +NAA	Multiple shoot formation Rooting	64
Phoenix dactylifera	Tree/ shrub	Leaves	Eeuwen's	NAA+ Sucrose	Callusing, Shoot initiation	92
Pistacia chinensis	Tree	Stem segments	½ DKW ½ WPM	BAP+NAA+IBA IBA+NAA	Multiple shooting Rooting	76
Pongamia pinnata	Tree	Nodal Segments	MS	BAP BAP+ GA_3 IBA	Multiple shooting Shoot elongation Rooting	113
Prunus armeniaca	Tree	Immature embryo, Cotyledon	MS	BAP+ 2,4-D	Callusing, Multiple shoot formation	28
Psophocarpus tetragonolobus	Climb	Nodal explants	MS IBA	BAP Rooting	Multiple shooting	51
Raphanus sativus	Herb	Cotyledon	MS	KN NAA	Multiple shooting Rooting	114
Ricinus communis	Shrub like herb	Shoot tip	MS	BAP+ IBA -	Multiple shoot formation Rooting	133
Saccharum officinarum	Graminoids	Meristem	MS ½ MS	2,4-D BAP+ NAA NAA	Callusing Multiple shooting Rooting	127
Salvadora oleoides	Tree	Shoot tip	MS	BAP+ NAA NAA	Multiple Shoot formation Rooting	68
S. persica	Tree	Nodal segments	MS	BAP+KN+ NAA IBA+NOA	Multiple shoot formation Rooting	130
Santalum album	Tree	Leaf disc	MS	2,4-D+TDZ TDZ+ GA_3	Somatic embryogenesis Plant regeneration	72
Sapindus mukorossi	Tree	Leaf	B_5	2.4-D+ BAP	Embryogenic callusing, Somatic embryo initiation	87

table 1. (continued).

Species	Habit	Explant used	Medium used	PGRs (mgl^{-1}) and other supplements	Result obtained	Reference
Sapium sebiferum	Tree	Nodal segments	MS	BAP+NAA	Multiple shoot formation	128
			½ MS	IBA	Rooting	
Sclerocarya birrea	Tree	Shoots, Hypocotyls, Epicotyls	MS	meta-topolin (mT)	Multiple shoot formation	57
				IBA	Rooting	
Sesamum indicum	Herb	Cotyledon, Hypocotyl	MS	TDZ +IAA	Multiple shoot formation	53
Simarouba glauca	Tree	Cotyledon	MS	BAP+ NAA	Callusing, Somatic embryo initiation	140
			½ MS	ABA	Somatic embryo maturation	
Simmondsia chinensis	Shrub	Nodal segments	MS	BAP+ Adenine	Multiple shoot formation	50
				IBA + IAA +NAA +AC	Rooting	
Sinapis alba	Herb	Cotyledon, Anther	MS	BAP+NAA, ZT+ NAA	Somatic embryo formation	86
				ZT+ NAA	Multiple shoot formation,	
		B_5		ZT+ NAA	Rooting	
Sorghum bicolor	Graminoids	Shoot tip	MS	BAP + KN + $AdSO_4$+ CW + NAA+ AsA	Multiple shoot formation	20
			½ MS	IAA	Rooting	
Sterculia foetida	Tree	Hypocotyl , Shoot tip	MS	BAP	Shoot multiplication	48
			½ MS	IAA	Rooting	
Tectona grandis	Tree	internodal segments	WPM	TDZ+IBA	Callusing	84
				BAP+GA_3	Shoot regeneration	
Terminalia bellerica	Tree	Seedling	MS	BAP	Multiple shoot formation	115
			¼ MS	IBA	Rooting	
T. catappa	Tree	nodal segments	MS	BAP+KN	Shoot multiplication	116
				IBA	*ex vitro* Rooting	
T. chebula	Tree	Cotyledon, Mature zygotic embryo	MS	2,4-D+KN	Callusing	31
				BAP	Germination of SE	
Theobroma cacao	Tree	Nodal, Apical stem	½ DKW	TDZ	Shoot multiplication and elongation	24
			MS	IBA	Rooting	

table 1. (continued).

Species	**Habit**	**Explant used**	**Medium used**	**PGRs (mgl^{-1}) and other supplements**	**Result obtained**	**Reference**
Thevetia peruviana	Tree	Leaf	MS	2,4-D+ KN	Callusing	38
				2,4-D+ KN + $AdSO_4$	Shoot initiation	
				$AdSO_4$+BAP	Elongation of shoot	
				IBA	Rooting	
Vernonia cinerea	Herb	Leaf, Nodal	MS	BAP+ NAA	Multiple shoot formation	49
			½ MS	IAA	Rooting	
Zanthoxylum bungeanum	Shrub/ tree	New stem	MS	BAP+NAA+GA_3	Multiple shoot formation	131
			½ MS	BAP+IAA	Rooting	
Zea mays	Graminoids	Embryo	½ MS,	Fructose, Sucrose, Maltose	Shoot elongation & rooting	39
Ziziphus spina-christi	Tree	Shoot tips and Stem nodal	MS	BAP	Multiple shoot formation	23
				IBA	Rooting	

Abbreviations used:
2,4-D-2,4-Dichlorophenoxy acetic acid
IBA- Indole-3-butyric acid,
$AdSO_{4}$- Adenine sulphate
AC- Activated charcoal
CH- Casein Hydrolysate
NOA- beta-Naphthoxyacetic acid
MS- Murashige and Skoog's (1962) nutrient medium
OM- Olive medium (Rugini 1984)
DKW-Driver and Kuniyuki medium (Driver and Kuniyuki, 1984)
KN-Kinetin
AsA- Ascorbic acid
ABA-Abscisic acid
TDZ- Thiadiazuron
IAA- Indole-3-acetic acid
NAA- Naphthalene acetic acid
CW- Coconut Water
$AgNO_3$- Silver Nitrate
AD- Adenin, 2ip-6-(gamma,gamma-Dimethylallylamino) purine
SE- Somatic embryogenesis
B_5- Gamborg's medium (Gamborg *et al*., 1968)
WPM, Woody plant medium (Loyd and McCown,1981)
Y3 medium or Eeuwen's medium (Eeuwen's, 1976)
ZT- Zeatin
GA_3- Gibberellic acid
BAP-6-benzyl amino purine

(Table 1). Physiological effect of GA_3 on plant is well-known and it is used as a medium for multiplication.

Auxins are the most studied plant growth regulators and have been shown to be involved in controlling fundamental aspects of plant development such as cell fate determination, cell division as well as cell polarity [119, 120]. It is known that a balance between auxin and cytokinin normally induces effective organogenesis. Though the nature of interaction between the two plant growth regulators is still not completely understood, cell division seems to be regulated by their interactions affecting different phases of cell cycle. While auxins are known to exert an effect on DNA replication, cytokinin exerts some control over the events leading to mitosis [121].

BAP in combination with NAA produces multiple shooting in *Beta vulgaris* [47], *Brassica napus* [45], *Broussonetia papyrifera* [122], *Camelina sativa* [123], *Celastrus paniculatus* [124], *Citrullus colocynthis* [125], *Gossypium hirsutum* [42], *Helianthus annuus* [81], *Hevea brasiliensis* [126], *Madhuca longifolia* [37], *Nicotiana tabacum* [66], *Saccharum officinarum* [127] , *Salvadora oleoides* [68] , *Sapium sebiferum* [128] and in *Vernonia cinerea* [49], (Table 1). However, combination of BAP and NAA with other growth regulators like KN, GA_3, IBA and Triacontanol has also produced multiple shoot formation in *Euphorbia tirucalli* [129], *Moringa oleifera* [58], *Oryza sativa* [71], *Pistacia chinensis* [76], *Salvadora persica* [130] and in *Zanthoxylum bungeanum* [131] (Table 1). In *Macadamia integrifolia* combination of BAP, GA_3 with IBA also produces multiple shooting [63] (Table 1).

BAP in combination with other auxin viz. IBA, IAA and 2,4-D has also produced multiple shooting in *Arachis hypogaea* [65], *Cucurbita foetidissima* [132], *Eruca sativa* [62], *Lupinus mutabilis* [46], *Melia azedarach* [44], *Momordica dioica* [67], *Prunus armeniaca* [28] *and Ricinus communis* [133] (Table 1). The combination of three growth regulator viz. BAP, IAA and IBA gave higher multiplication rate in *Miscanthus giganteus* [105] (Table 1).

Combination of other cytokinins like TDZ, ZT and auxin viz. IBA, NAA, IAA stimulates shoot proliferation in *Calendula officinalis* [52], *Carthamus tinctorius* [134], *Linum usitatissimum* [56], *Sesamum indicum* [54] and *Sinapis alba* [86], (Table 1).

Application of additives is adapted to the cultural needs i.e. objectives of the experimental studies like micropropagation, regeneration, cytodifferentiation, androgenesis, biosynthesis of secondary metabolites and biotransformation of cells as well as the particular plant species taken [135]. Some additives such as adenine sulphate, putrescine in combination with BAP, 2iP and IAA produced profuse regeneration and multiplication in *Azadirachta indica* [136] (Table 1). However, adenine sulphate in combination with KN and NAA also produces multiple shooting in *Balanites aegyptiaca* [27] (Table 1). Sometime BAP in combination with KN, NAA, $AdSO_4$, coconut water and ascorbic acid yielded shoot multiplication in *Sorghum bicolor* [20] (Table 1). Other additive such as $AgNO_3$ in combination with BAP and NAA produces multiple shoots in *Brassica juncea* [79] (Table 1).

Other than these classical plant growth regulators, new natural growth substance viz. polyamine [137] with regulatory roles in tissue culture has been discovered in last few years. BAP in combination with polyamine has produced multiple shooting in *Corylus avellana* [89] (Table 1).

Amino acids are important for growth regulation as well as modulators of growth and cell differentiation, which may be affecting general metabolism and consequently morphogenesis [138]. In one case combination of cytokinin, auxin, additive and amino acid produced multiple shooting in *Jatropha curcas* [40] (Table 1).

In another study, Peptone in combination with BAP and 2, 4-D produced multiple shooting in *Persea americana* [64] (Table 1). Previously, peptone was added as the carbon and nitrogen source for plant tissue culture. It has been suggested that at an efficient concentration, organic and inorganic nitrogen sources can promote the growth of explants [139]. While in some cases BAP in combination with NAA also produces somatic embryogenesis in *Garcinia indica* [32], *Simarouba glauca* [140] and *Sinapis alba* [86] (Table 1).

Physical factors

It is well known that both hormonal and physical factors are necessary for maintaining tissue culture.

Light

Perusal of literature indicates that light intensity plays an important role for satisfactory shoot growth and multiplication. Different researchers used different time and intensity of light for their purpose of culture incubation. Some researcher reported that to reduce oxidation, cultures were kept in dark for the first 20-24 h in *Corylus avellana* [90] and for 7 days in *Olea europaea* [25] and then transferred under light. Some researchers maintained cultures for callus induction, germination of seeds in a growing room, in dark while for somatic embryo regeneration and multiple shoot induction generally culture were maintained under 16/8h photoperiod supplied by cool-white fluorescent tubes and 13-50 $\mu molm^{-2}s^{-1}$ photosynthetically active radiation (PAR). In *Annona squamosa* light intensity levels at 1000 to 4000 lux were used. Of all the light treatments 2000 lux (16 h light) resulted in rapid shoot bud initiation [60]. However, many researchers have incubated culture under 4.5 $\mu mol\ m^{-2}s^{-1}$, 35 $\mu mol\ m^{-2}s^{-1}$, 60 $\mu mol\ m^{-2}s^{-1}$, 40 $\mu mol\ m^{-2}s^{-1}$ and 25 $\mu mol\ m^{-2}s^{-1}$ photon flux density for 10-15 h photoperiod in *Azadirachta indica* [136], *Brassica juncea* [79] *Melia azedarach* [44], *Momordica dioica* [67], and in *Persea americana* [64] which is slightly less. Some workers reported that for shoot proliferation initially a light intensity of 40 $\mu mol\ m^{-2}s^{-1}$ was used while 20 $\mu mol\ m^{-2}s^{-1}$ was used for subculture [104]. Reducing the light intensity from 40 to 20 $\mu mol\ m^{-2}s^{-1}$ increased the rate of shoot multiplication in *Cynara cardunculus* while in *Sinapis alba* the frequency of shoot regeneration was declined with reduction in the photoperiod to 16 h [86]. In *Myristica malabarica* all cultures were incubated in continuous light with a photosynthetic photon flux density of 35 $\mu mol\ m^{-2}s^{-1}$ provided by 40W cool white, fluorescent tubes [35].

Temperature

Many researchers have used an optimal temperature of 25°C ± 2°C for shoot multiplication, while some researchers has used lower temperature such as 20-24°C in *Lupinus albus* [46], 22°C in *Linum usitatissimum* [55, 123], 22.5°C in *Olea europaea* [25] and 23°C in *Corylus avellana* [90]. However, other researchers have used slightly higher temperature like 28°C in *Hibiscus sabdariffa* [54], 30°C in *Gossypium hirsutum* [42, 91]. Few researchers have used lower temperature at night in comparison to 30°C /24°C and 25°C /20°C (day/night) [81, 110].

Relative humidity

Relative humidity (RH) is a major factor in enhancing the biochemical, physiological and morphological characters of plantlets during *in vitro* acclimatization when transplanted to *in vivo* conditions [141]. Generally plantlets were acclimatized under different relative humidity from 50-60 % in *Aegle marmelos* [41], *Eruca sativa* [62], *Momordica dioica* [67], *Salvadora oleoides* [68], *Sorghum bicolour* [20], *Thevetia peruviana* [38] and *Vernonia cinerea* [49] to high humidity of 70-80 % in *Azadirachta indica* [136], *Jatropha curcas* [40], *Persea americana* [64] and *Saccharum officinarum* [127].

Rooting of *in vitro* regenerated shoots

For any micropropagation protocol, successful rooting of *in vitro* regenerated shoots is a prerequisite to facilitate their establishment in soil. In trees and shrub the rhizogenesis has been found to be very difficult. Considerable work has been done to enhance rooting efficiency in oil yielding plants. Rooting of *in vitro* regenerated shoots can be accomplished both under *in vitro* and *ex vitro* conditions.

Variation in rooting response may be affected by different conditions of the shoots used for root induction, variations in the medium used for multiplication before root induction, the number of subcultures before root induction and the culture period on multiplication medium before transfer to root induction medium. The differences in rooting response may be a result of genotype or cultural conditions [80].

***In vitro* rooting of shoots**

The *in vitro* rooting capacity depends on the interaction of internal and external factors such as medium, form of medium, genotype, carbohydrate, growth regulators etc.

Genotypes/Cultivars

According to some authors rooting response in

Cucurbita pepo [80] and *Olea europaea* [25] was genotype/cultivar dependent. In *Cucurbita pepo* most of the shoots had developed roots before fourth week. Overall, cultivar Bulum had a better rooting response (88 %) than Rumbo (80 %) and in *Olea europaea* AOC variety gave root initiation on OM medium in combination with IBA and Laragne genotype gave rooting in WPM in combination with NAA. Gurel *et al.* [47] reported that breeding line M114 in *Beta vulgaris* resulted in better response in terms of rooting than ELK345 and M1017. However, Hazra *et al*. [42] achieved rooting on basal MS medium, there were no significant differences in rooting percentages among the various genotypes of *Gossypium hirsutum* which were tested.

Medium

Out of various media used MS medium has been most commonly used for root induction. Most authors reported that the use of the full strength MS medium [23, 44, 58, 110, 123] with major elements reduced to one quarter [22] to half strength [49, 67,129] was best for root induction (Table 1). Some authors used full strength WPM reduced to half strength WPM [69] and modified WPM [56] for root induction. In some cases White's medium [60] OM medium [25] and Eeuwen's medium [92] (Table 1) were also used for root induction.

Form of medium

Both liquid as well as solid media was used for root induction. Some researcher found that liquid medium was more effective than solid medium. The root development on solid medium takes longer time than liquid medium. Roots that regenerated on solid medium were thin and long and were easily loosened during acclimatization. In contrast, roots regenerated in liquid media were thicker and healthy as reported in *Raphanus sativus* [114].

Carbohydrate

Root formation is an energy demanding process and requires exogenous supply of carbohydrates. In addition, growth and root initiation are highly energy requiring processes that can occur at the expense of available metabolic substrates, which are mainly carbohydrates [142]. However, this being the last stage of *in vitro* culture, it is important to transform the plant from heterotrophic to auto-trophic mode of nutrition. Thus, the supply of exogenous sugars should be reduced at this time. Generally 2-3 % of sucrose was used while in *Phoenix dactylifera* 4-9 % of sucrose was used for rooting [92]. The fact is that, sucrose can be the cause of root initiation.

Growth regulators

Growth regulators are essential for root morphogenesis but some time well developed roots were also found on hormone free medium. Various researchers [66, 45, 79, 54,126,133, 111] have used hormone free MS media for rooting (Table 1). This condition is possible due to a high content of endogenous auxins in plant. However, auxins viz. IBA, IAA and NAA were used alone as well as in combination for root induction. It has been established that auxin stimulates lateral root initiation by activating quiescent pericycle cells to initiate division and then expansion which facilitate lateral root emergence [143]. Therefore, appropriate synthesis, signaling and transport of auxin are required for root formation [144]. IBA is most commonly used auxin used for rooting in various plants ex. in *Azadirachta indica* [136], *Broussonetia papyrifera* [122], *Calophyllum apetalum* [22], *Carya illinoinensis* [56], *Cerbera odollam* [26], *Cucurbita pepo* [80], *Madhuca longifolia* [37], *Melia azedarach* [44], *Momordica dioica* [67], *Olea europaea* [25], *Pongamia pinnata* [113], *Psophocarpus tetragonolobus* [51], *Sclerocarya birrea* [57], *Theobroma cacao* [24], *Thevetia peruviana* [38] and in *Ziziphus spinachristi* [23] (Table 1). However, some researchers found that IAA was also suitable for root induction in *Sorghum bicolor* [20], *Sterculia foetida* [48] and in *Vernonia cinerea* [49] (Table 1). Some workers have also used NAA for inducing rhizogenesis in *Arachis hypogaea* [65], *Beta vulgaris* [47], *Calendula officinalis* [52], *Carthamus tinctorius* [134], *Cleome viscosa* [59], *Euphorbia lathyris* [110], *Helianthus annuus* [81], *Phoenix dactylifera* [92], *Raphanus sativus* [114], *Saccharum officinarum* [127] and in *Salvadora oleoides* [68] (Table 1).

Rooting of *in vitro* regenerated shoots was also achieved by two-step procedure dipping the cut ends of shoots for a few hours in auxins such as IBA, NAA and IAA in *Simmondsia chinensis* [50], IBA in *Jatropha curcas* [40], *Melia azedarach* [44] and *Sapium sebiferum* [128] (Table 1) instead of being continuously cultured on auxin containing medium and then transferred in growth regulator free medium. It is an established fact that although auxins are essential for root induction but they are not required for root growth. Instead of that there continued presence may even inhibit the root growth [145]. Therefore, after pulse treatment for 24-48 h shoots were subsequently placed on hormone free medium for rooting.

Combination of growth regulators (IBA and NAA) were also used for induction of roots in *Pistacia chinensis* [76]. In *Persea americana* peptone was used in combination with NAA, IAA and IBA [64]. In *Moringa oleifera* two auxins viz. IAA and IBA were used [58]. In *Cannabis sativa* [70], *Garcinia indica* [32] and *Euphorbia tirucalli* [129] combinations of NAA and IBA were used. However, in some cases cytokinin BAP or KN were also used in combination with IAA or IBA for the induction of rooting in *Zanthoxylum bungeanum* [131] and *Eruca sativa* [62] (Table 1).

In *Prunus armeniaca* the best rooting percentages were induced by NAA while the largest number of roots per shoot were obtained after induction with IBA[28] (Table 1) while in *Macadamia integrifolia* even after all the auxins treatment, rooting was not observed [63].

Activated charcoal

Supplementation of activated charcoal (AC) to the culture medium was found to have a remarkable positive influence on the rooting efficiency of cultured shoots. Since AC has the ability to adsorb deleterious compounds released by explants into the medium, it was added to the rooting medium to improve root growth. AC was used alone or in combination with auxins viz. IAA, IBA to produce roots in *Aegle marmelos* [41], *Annona squamosa* [60], *Balanites aegyptiaca* [27], *Miscanthus giganteus* [105], *Momordica dioica* [67] and in *Simmondsia chinensis* [50] (Table 1).

ex vitro rooting of *in vitro* regenerated shoots

Attempts have also been made for root induction under *ex vitro* conditions such as in *Momordica dioica* [67] where a moderate success was achieved in inducing *ex vitro* rooting of the shoots when the shoots were pulse treated with IBA (Table 1). Only 34 % of the pulse-treated shoots were rooted within 4 weeks in greenhouse conditions. However, in *Terminalia catappa* 80 % rooting of cultured shoots treated with IBA for 4 mins [116] (Table 1) was obtained. In *Tectona grandis* rooting was obtained in soil and sand (1:1) [84]. There is further scope for increasing the frequency of *ex vitro* rooting so as to make protocol less expensive [130].

Comparison of *in vitro* and *ex vitro* rooting

A comparison of *in vitro* and *ex vitro* rooting has been observed in *Celastrus paniculatus* [124] (Table 1). They indicated that among the various rooting trials, *ex vitro* rooting of shoots with simultaneous hardening was most efficient. The method standardized in the present study is simple as it has eliminated separate steps for *in vitro* rooting and hardening. Whereas in *Momordica dioica* moderate success was achieved in *ex vitro* rooting [67] (Table 1).

In vitro flowering

In vitro flowering serves as an important tool in studying flower induction, initiation and the floral developmental process by utilizing plant growth regulators such as cytokinins, gibberellins and auxins [146]. It is known that during the change from vegetative to the flowering state, the growth correlations within the apical meristem of a shoot are changed, which leads to the loss of apical dominance. Apical dominance is under hormonal control with auxins, cytokinins and gibberellins having a sequential role [147]. Sharma *et al.* [62] reported that the flowers were initiated in the media containing auxin (IBA) and cytokinin (BAP, KN) in combination (Table 1). Thus, it is evident that the presence of auxin and cytokinin is essentially required for the induction of flowering in *Eruca sativa in vitro*, as no inflorescences were observed in the control explants devoid of cytokinins while Baghel and Bansal [111] achieved *in vitro* flowering in *Guizotia abyssinica* on the media containing GA_3 and BAP in combination (Table 1).

Acclimatization and field establishment

Prolific rooting on *in vitro* grown microshoots is critical for the successful establishment of these shoots in the greenhouse or field. Various procedures for hardening plants have already been described. All are based on the principle of gradually reducing the humidity around the rooted plantlets and altering plant metabolism from partial dependence to full independence of an external carbohydrate source. The particular conditions reported in different papers probably reflect the climatic conditions of the region, season and the facilities that were available.

Traore *et al.* [24] reported in *Theobroma cacao* that the roots were transplanted into pots containing a moist soil mix consisting of equal parts of promix as well as concrete sand and then transferred then maintained in green house at 80 %. Natural light was supplemented with high pressure sodium lamps as needed to maintain a minimum of 250 µmol $m^{-2}s^{-1}$, while automatically retractable shading limited light levels to a maximum of 1400 µmol $m^{-2}s^{-1}$.

Many researchers [23, 38, 62] reported that plantlets of *Eruca sativa*, *Thevetia peruviana* and *Ziziphus spina-christi* with roots were potted in small polycups containing sterilized soil and vermicompost 3:1. The plantlets were then hardened by keeping the plantlets covered with inverted glass beaker onto the polycups to maintain high humidity. Finally, the plants were transplanted in the natural environment. About 90 % of the plantlets survived after the acclimatisation process.

Seetharam *et al.* [49] reported that healthy plantlets of *Vernonia cinerea* with roots in the tubes were kept open for 5-6 days by loosing cotton plugs. They were transferred to plastic cup with sterile inert supporting soilrite. Each plant was covered with a glass beaker and maintained in the growth chamber at 90 % RH with 14 h photoperiod. The well developed plants were transferred to soil mixture and 90 % RH was gradually reduced to 60 % over 20 day. Regenerated plantlets were then transferred to nursery successfully with 80 % survivability.

Loganathan *et al.*[36] reported that the plantlets of *Glycine max* (5-6 cm) with well-developed shoots and roots were transferred to pots filled with 3:1 mixture of sandy loam soil and farmyard manure (FYM), while in *Sterculia foetida* [48] rooted plantlets were placed in liquid MS basal medium (quarter strength), transferred to pots containing sterilized sand soil, manure mixture (1:1:1) and liquid MS basal medium (half strength) were irrigated to these plants. The plants were covered with polythene bags to maintain high relative humidity and maintained in a greenhouse under natural light conditions until seed harvest and 40 % plant has survived.

Baskaran and Jayabalan [20] achieved 72.4 % survival of plantlets in *Sorghum bicolour* after hardening on garden soil, farmyard soil and sand (2:1:1).

Singh *et al.* [50] reported that rooted plantlets of *Simmondsia chinensis* were transferred to culture bottles filled with sterile sand and moistened with half-strength MS liquid medium. Rooted plantlets in the greenhouse subjected to different temperature, humidity regimes, temperature of 25-30°C and relative humidity 80 % were found to be ideal for plant establishment (99 %). Minimum survival (2 %) was observed.

Moyo *et al*.[57] reported that after 8 weeks in culture, *in vitro* rooted plantlets of *Sclerocarya birrea* were planted in plastic containers in 1:1 (v/v) vermiculite: sand mixture and placed in an environmentally controlled mist house for 4 days for acclimation *ex vitro*. A high pressure fog system was used to maintain high relative humidity between 90 and 100 %. The average midday photosynthetic photon flux density (PPFD) in the mist house was 30-90 l mol $m^{-2}s^{-1}$ under natural photoperiod conditions. The plantlets were transferred to a greenhouse in which the temperature was maintained at 25±2°C under natural photoperiod conditions and a mid day PPFD of about 400-1,800 l mol m^{-2} s^{-1}.

Behera and Sahoo [127] reported that the plantlets of *Saccharum officinarum* were transferred to plastic trays for hardening which contain autoclaved garden soil, farmyard manure and sand (2:1:1). The hardened plantlets in the plastic trays were covered with porous polyethylene sheets for maintaining high humidity and kept under shade in a net house for further growth and development.

All were irrigated with 1/8 MS basal salt solution devoid of sucrose and inositol every 4 days for 2 weeks. After 30 days, the plantlets were transplanted into the soil in field conditions. The plantlets with well developed shoot and roots after acclimatization were successfully transplanted in soil with 85 % acclimatization of survivability potential

Siril and Dhar [128] reported that the rooted plantlets of *Sapium sebiferum* were washed in tap water and transplanted into polyethylene pots containing soil: vermiculite (1:1). Potted plants were grown in a growth chamber at 25±1°C and under a 16 h photoperiod with a light intensity of 40 µE $m^{-2}s^{-1}$provided by cool white fluorescent tubes. Plantlets were covered with polyethylene bags for the first 2 weeks to maintain humidity. They were watered every other day with quarter-strength MS mineral salts. Plants were transferred to the field after 4 months of *ex vitro* growth (1 month in the growth chamber and 3 months in the greenhouse).

Bele *et al.* [72] reported in *Santalum album* that the plantlets were planted in 2.5 cm root trainers filled with 1:1:1 sand, soil and FYM sterilized mixture. Root trainers with transplanted plants were placed in Environmental Growth Chamber under 30±2°C and 65±5 % RH for 15-20 days for acclimatization. Acclimatized plants were then transferred to Green House for 30 days for hardening before transplanting them into the field. Nahar and Borna [133] and Baghel and Bansal [111] reported that the regenerated plantlets of *Ricinus communis* and *Guizotia abyssinica* were respectively transferred to plastic cups containing sterile soil, sand, compost (1:1:1), covered with polythene and maintained in tissue culture conditions. Finally the developed plantlets were transferred to the field.

Laura *et al.*[68] reported that the regenerated plants were taken out from medium and transplanted in a pot containing sterile soil and vermiculite (1:1) mixture. Initially the plants were kept in laboratory conditions. All plants were watered with quarter strength MS salt solution on alternate days for 2 weeks and finally plants were shifted to polyhouse followed by field conditions. Then plants were transferred to poly house for 20 days to ensure acclimatization. After acclimatization in polyhouse, plants were transferred to the field conditions with 80 % survival rate. The protocol reported in this study can be used for rapid and large scale multiplication of true to type plants

Naik and Naik [51] reported that the plantlets were transplanted into plastic pots containing soil mix and irrigated with tap water regularly. The plantlets were covered with polythene bags to maintain high humidity and acclimatized at 25± 2°C. After 10 days the polythene bags were removed and plantlets were transferred to a green house. About 60 % of *in vitro* proliferated plantlets survived. After 5 weeks the regenerated plantlets were transferred to soil under green house condition.

Kim *et al.*[114] reported in *Raphanus sativus* that after 4 weeks, regenerated plants with well-developed roots were acclimatized in water for 7 days and then transferred to soil.

Li *et al*.[76] reported in *Pistacia chinensis* that the rooted plantlets with three to four roots and well-developed leaves were transferred to pots containing sand, chernozem type soil and covered with plastic cups. Normal growth of the potted plants was observed after 3-4 weeks of trans-plantation. About 60 % of rooted plantlets survived after transplantation to soil and grew to maturity in the greenhouse. When compared with the donor plants, the regenerated plants did not show any visible variation in morphological or growth characteristics. The rooted plantlets were then transferred to a shade house where they could grow well after acclimatization.

Sugla *et al.* [113] reported in *Pongamia pinnata* that the well-rooted plantlets were washed thoroughly in running tap water before being transplanted into plastic pots containing sterilized soil and vermiculite (1:1) Plants were covered with transparent polyethylene bags to maintain adequate moisture and then transferred to the green-house (28°C d/20°C night, 16 h d-length, 70 % relative humidity). After a week, the plastic covers were gradually removed and the plantlets were maintained in the greenhouse in earthen pots containing normal garden soil until they were transplanted to the nursery. Ninety-two percent of the plantlets transferred to soil and vermiculite survived, while 98 % of the plants transferred to soil survived. Plants were gradually exposed to low humidity conditions and finally kept in an open nursery. The plants were finally transferred to the field.

Isutsa [118] reported that plantlets of *Passiflora edulis* were transplanted to the same sand: soil mixture as that used for *ex vitro* rooting and acclimatized in a greenhouse.

Shekhawat *et al*.[67] reported in *Momordica dioica* that *in vitro* rooted shoots were gradually hardened by transferring them to bottles containing soilrite with habitat soil under 70 % RH at 28 ± 2°C temperature in the greenhouse for 30 days.

Saini *et al*.[58] reported in *Moringa oleifera* that hardening was done in plastic bags containing autoclaved mixture of soil, sand and vermi-compost (3:1:1 v/v). Plants were watered, then covered with transparent polythene bags and kept under partial sunlight inside a greenhouse at ambient temperature (26–28°C). After 15 days, the polythene bags were removed and the survived plants were maintained inside the greenhouse for another 15 days. These hardened plantlets were transferred into the field.

Some researchers [32, 40, 42] that plantlets of *Garcinia indica*, *Gossypium hirsutum* and *Jatropha curcas* with well developed roots were transferred to plastic cup containing autoclaved sand as well as soil (1:1) and maintained in the same environmental conditions for 1 week. In *Jatropha curcas* the plantlet were watered regularly with 1/10th strength MS liquid media, subsequently plantlet were transferred to earthen pots containing coarse sand as well as garden soil (1:1:2) and kept in shade for 2 weeks before transferring them to the experimental garden soil (1:1:2) and kept in shade for 2 weeks before transferring them to the garden.

Leyva *et al*. [54] reported in *Hibiscus sabdariffa* that the plantlets were transferred into growth jars with a sterile mixture of perlite, sunshine (1:1) and 50 % liquid MS without sucrose. A cellophane cover was placed to promote the gaseous exchange, after 3 weeks in lab condition the cover was removed and the plantlets were transferred to pots containing sterile soil and transferred in a green house. After 4 weeks they were transfer to field.

Ripley and Preece [110] reported in *Euphorbia lathyris* that rooted shoots were then potted into peatlite medium in plastic pots placed upon moist paper towels for 1 week. Plants were then directly placed onto greenhouse benches with no further special care.

Lan and Yoeup [132] reported that plantlets of *Cucurbita foetidissima* were transferred to pots containing sterile vermiculite. Each pot was enclosed in a polyethylene bag after watering and maintained in a growth chamber at 25 ± 1°C under 16-h illumination (45 mol $m^{-2}s^{-1}$) with fluorescent lamps. Bags were progressively opened weekly. After 3 weeks of acclimatization, plantlets were transferred to large pots for further growth with plantlets transferred to soil pots after 2 weeks of initial hardening under culture-room conditions. Almost 70 % of these regenerants survived and showed new branch development

Sakr *et al*.[26] observed that the plantlets of *Cerbera odollam* were successfully lifted when they were transferred in a mixture of peatmoss and sand (1:0, 1:1; 1:2 and 1:3 respectively) and covered by polythene sheets in greenhouse. Approximately 40 % of plantlets survived.

Meena *et al*. [125] reported that the plantlets of *Citrullus colocynthis* with 6-7 leaves and well developed root system were removed and transferred to pot containing soilrite. These pots were kept in growth chamber for 15 days at 26 ± 2°C and 2000 lux intensity for acclimatization. In order to maintain high humidity, the pots were covered with inverted glass beaker. After six months when new leaves emerged from these plantlets, they were taken outside the growth chambers and kept in shady place under normal temperature and light. A 60 % survival rate was obtained when acclimatized plantlets were transferred to green house.

Anburaj *et al*. [59] reported in *Cleome viscosa* that the regenerated plants were successfully trans-ferred to earthen pot containing soils. Almost all (90 %) the *in vitro* regenerated plants successfully survived in green house conditions. Similarly, the success of transplantation was 85 % when plantlets were sufficiently healthy with new growth. They were subsequently transferred to larger pots and gradually acclimated to outdoor conditions.

Radhika *et al*.[134] reported that the shoots of *Carthamus tinctorius* with capitula were transferred to sterilized vermiculite saturated with a solution of 0.5 mg dm^{-3} NAA and maintained under high humidity for a week by covering the pots with polythene bags. The plantlets were maintained in the growth room for another week before getting transferred to pots. The survival frequency of rooted shoots was 32.4 % while that of the rootless micro shoots was 18.1 %.

Thengane *et al*.[69] reported in *Calophyllum inophyllum* that the rooted plantlets were transferred to sterilized potting mixture of soil, cocoa peat as well as sand (1: 2 : 1) and then acclimatized in a greenhouse with the temperature of 25 ± 2°C, 80 % relative humidity and with 77 % survival rate after a period of 5 weeks. The well developed and hardened plants after 8 weeks were transferred to earthen pots containing a mixture of garden soil and farmyard manure (1:1) for further growth and development and finally planted in the institute campus.

Wang *et al*. [70] reported in *Cannabis sativa* that the well growth *in vitro* plantlets, after treatment with 0.2 % (w/v) Bavistin, were hardened by the following two-steps procedure: (i) controlled conditions of culture room for 3-4 weeks and (ii) semi controlled conditions in the shade house for 2 weeks allowed 95 % of plantlets to acclimatize, after which 99 % of these plantlets were surviving for 3 months after getting transferred to the field. Renukdas *et al*.[56] reported that in *Carya illinoinensis* the plantlets were initially transferred to peat pellets and subsequently to the greenhouse. Ghnaya *et al*.[45] reported in *Brassica napus* that when hardened just after rooting, regenerated plantlets transferred to pots in greenhouse showed a high rate of survival upon acclimatization (80-100 %) The plants developed until flowering 8 weeks later and fertile seeds were harvest.

Arora *et al*. [136] reported in *Azadirachta indica* that *in vitro*-rooted shoots (plantlets) were hardened first in an inorganic salt solution under culture room conditions for about 30 days and then grown in potted soil under glasshouse conditions and after about 6 months of growth, they were transplanted in the field. The root-regenerated plants in field showed uniform luxuriant growth.

Binet *et al*.[25] reported that micropropagated olive plantlets (Aglandau, Tanche and Laragne) were transplanted at four-to-five node stage to pots containing a 60:40 (v/v) mixture of sterilized peat and Perlite (Puteaux SA, Les Clayes sous Bois, France). Pots were maintained in the glasshouse for 7 days in a mist unit with a transparent polyethylene lid. During the subsequent 15-20 days, the polyethylene lid was progressively removed to reduce humidity. Plants were further maintained in the glasshouse under natural photoperiod conditions. For all genotypes, *in vitro* regenerated plantlets were acclimatized in a sterilized substrate containing 60 % peat and 40 % Perlite (v/v) in a mist unit in the glasshouse. The percentages of survival were 92, 70 and 57 for Aglandau, Laragne and Tanche plants respectively. These results illustrate a genotype-dependent behaviour towards acclimatization for *O. europea* plants. In addition, the experimental conditions required for survival of Tanche and Laragne olive plantlets have been defined.

Somatic embryogenesis

Many researchers have worked in callus formation and somatic embryogenesis on oil yielding plants [148-154]. Different explants such as zygotic embryo, mature embryo, cotyledon, hypocotyl, plumule, cotyledonary node, leaf, stem segments, immature inflorescence explants and anther even nucellar tissue have been used for somatic embryo induction in oil yielding plants. Different PGRs were used in the medium for embryo induction, embryo germination and maturation such as auxins (2-4,D, IAA, IBA, NAA), cytokinins (2-iP, BAP, TDZ, KN, ZT), additives (CW, CH, AC, ABA, TRIA, proline), GA_3, picloram and nitrogen source (asparagine, glutamine). Varied concentrations of PGRs were used for somatic embryogenesis. However, in *Elaeis guineensis*, where immature zygotic embryos (IZEs) were used as explants, no PGRs were used in the medium for embryo induction and embryo germination.

Conclusion and future prospects

Micropropagation is an ideal method to make full use of medicinally and other important plant species. Compared with the sexual progeny, clonal ancestors can keep the original integrity of plant species, narrow the difference among individuals, maintain their stability and seed yield. Further, the delivery and effective distribution channels plays major role in the commercial production of micropropagated plants. In conclusion, the present efficient and reliable plant regeneration protocol can be potentially utilized for ex-situ conservation and mass propagation of oil yielding plant clones to meet the growing demand of energy plantations as well as need of herbal industry for therapeutic purpose. Using this technique, it is possible to produce healthy and disease free clones which could be released to their natural habitat in large scale. In view of these, the present protocol provides a useful system in plant breeding and crop improvement. It can also be used for the study of physiological signals that induce *in vitro* flowering.

Acknowledgments

One of us (S. Baghel) acknowledges funding received under the scheme Rajiv Gandhi National Fellowship (RGNF) of University Grants Commission (UGC), New Delhi India for carrying out this research.

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