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RESEARCH ARTICLE

A Review on *In vitro* Propagation of Turmeric (*Curcuma longa* Ln.)

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Abstract: Turmeric (*Curcuma longa* Linn.) is an important medicinal and spice crop which belongs to the family Zingiberaceae. Rhizomes are the commercially valuable part of the plant, commonly used as planting material in conventional propagation. Normally large portion (20-25%) of good quality rhizomes must be allocated as planting materials from the fresh harvest. Higher susceptibility to soil borne diseases is also a problem in using rhizomes as planting materials. Therefore, development of novel propagation techniques is important. The *in vitro* propagation has been considered as an effective alternative method for rapid regeneration of turmeric. Based on the above facts it is important to review the *in-vitro* propagation studies conducted in worldwide in order to identify best *in-vitro* propagation protocol suitable for local condition. In this regard, explant sterilization is a crucial factor; 70% ethanol, Carbendazim (fungicide), mercuric chloride and Clorox (commercial bleach) are mainly used to sterilize sprouting rhizome buds. Hormones such as BAP (6-Benzylaminopurine), BA (Benzyl Adenine), Kn (Kinetin) alone or in combination with NAA (Naphthalene Acetic Acid) or IAA (Indole-3-Acetic Acid) are commonly used in shoot multiplication as they play a vital role in bud production (2.0-4.0 mg/l of BAP were found as best hormone concentrations for shoot initiation). Roots were mainly induced by auxins like IBA (Indole Butyric Acid), IAA and NAA (2.0mg/l IBA). When acclimatization was done properly with Sand: Soil: Peat (1:1:1) combination as the potting media 70-90% of survival rate could be observed from regenerated plantlets.

Keywords*: in vitro regeneration, Shoot induction, Sterilization, Root induction*.

Introduction

Turmeric (*Curcuma longa* Linn.), which is an important spice on the Asian culinary is a rhizomatous herbaceous perennial plant. It belongs to the Zingiberaceae family having many uses as a spice, coloring agent and a medicinal plant (Nasiruzzaman et al, 2005; Xiang et al., 2011). Pemba and Sharangi (2017) stated that Turmeric comprises of nearly 130 different species. The most common varieties of Turmeric are *Curcuma longa*, *Curcuma aromatica*, *Curcuma amada*, *Curcuma angustifolia* and *Curcuma zedoria* which reported from India while many are widely distributed throughout tropical and subtropical regions of the world. Among the species of turmeric, *Curcuma longa* is the most extensively used species. The word 'Curcuma' is derived from the Arabic word 'Kurkum' which gives the meaning of yellow color (Patil & Srinivasan, 1971).

Turmeric is reported to have originated from Southeast Asian region where the use of turmeric believed to be started back about 4000 years to the Vedic culture in India. Now, it is widely cultivated in many countries of the world including India, China, Taiwan, Indonesia, Sri Lanka, Brazil, Peru, and in many parts of the African and Australian continent. Major growing areas of turmeric in Sri Lanka are Kurunegala, Gampaha, Kalutara, Kandy, Matale and Ampara districts.

The conventionally used growing method for *Curcuma longa* is the rhizome propagation (Berni et al., 2014). Rhizomes are usually collected from the previous cultivation and only well matured good quality rhizomes are selected and store in dry cool place until sprouting, usually in April-May. Pre sprouted rhizomes with 1-2 buds are planted in the field on the onset of raining at the end of April or beginning of May. The weight of a rhizome which is

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suitable for planting is around 30-50g (Figure 1). A good irrigation facility should be provided to get a high yield. Rhizomes which have gained the correct level of maturity for field planting should be planted in raised beds or ridges with proper irrigation and drainage system during the main cropping season of March to April. These can be intercropped with Coconut like cultivations as well. Well drained soil such as Sandy loam soils are most suitable for growth of turmeric. Soon after planting they should be mulched with a suitable mulching material to retain moisture in the soil and avoid drying of the rhizomes. Finally, after 8 – 10 months of planting, harvesting could be initiated after it started the drying of the leaves.

Figure 1: Sprouting rhizome buds (Ernst & Durbin, 2019)

The growth, yield and yield quality of turmeric depends mostly on the soil fertility planting density, planting method and planting material (Mekonnen & Garedew, 2019; Kumar & Gill, 2010). It further describes that higher planting densities could produce higher yield, but number of rhizomes per plant decrease with increasing plant density. The use of mother rhizomes as planting materials has shown a significant higher value of yield when compared to primary and secondary fingers of turmeric rhizome.

Although conventional propagation (Figure 2) is through rhizomes, their multiplication rate is very low (Gurav et al., 2020). In a growing season only 10-15 lateral buds are producing from a single rhizome and some portion of this harvest (nearly 20 – 25%) should retain as planting materials for the next season to grow (Sinchana et al., 2020). There are several reasons involved with this problem of low yield and quality such as slow multiplication or regeneration rate, high susceptibility to soil fungal, bacterial and nematode diseases and they are difficult to propagate through seeds because of poor flowering and seed set (Zhao, 2020). So large quantity of planting materials as rhizomes are required for cultivation and due to this market share of the harvest reduce by 20-25%.

Figure 2: Conventionally propagated Turmeric plants established in a pot

In vitro propagation is a novel technique which uses the ability of any plant part to regenerate in to new and healthy plantlets under aseptic conditions in a controlled environment in a defined culture medium. Under these conditions of *in vitro* culture number of pathogen free planting materials could be obtained within a short time (Kumar & Reddy, 2011). Several studies have been conducted to develop a standardize protocol for *in-vitro* propagation of turmeric. Now a days there is a high demand for turmeric in the local market due to ban of turmeric importation by the government, but the supply does not meet the demand. One of the reasons may be unavailability of quality planting materials for large scale cultivation and about 20-25% of harvest must be used as planting materials. Therefore, there is a possibility to introduce plant tissue culture techniques to produce turmeric plantlets to meet the growing demand for planting materials from farmers. Accordingly, this paper aims to review a suitable protocol for *in vitro* regeneration of turmeric with optimum conditions so that to develop large number of disease-free healthy plantlets that could be obtained for the field cultivation.

***In vitro* micro-propagation**

Many studies have been used rhizome buds as *ex-plant* in micro-propagation of *C. longa* (Rahman et al., 2004; Tyagi et al., 2004; Tyagi et al., 2007; Das et al., 2010; Soundar Raju et al., 2015; Yee et al., 2019). Even though, the extensively used *ex-plants* are the rhizome buds, some scientists have used immature inflorescence also (Salvi et al., 2001). It was found that these inflorescence explants could be successfully used to differentiate plants under *in vitro* conditions. However, rhizome buds have high susceptibility to contaminate with soil borne diseases and the rate of regeneration is very low under conventional vegetative propagation methods. To overcome these problems, *in vitro* regeneration was found to be a good solution with proper selection of a source of explants, explant types, sterilization method, plant growth medium, growth regulators and culture conditions (Figure 3). With the regulation of these conditions, *in vitro* culture would give successful regeneration of mass produce of the disease-free plantlets (Haque et al., 2009).

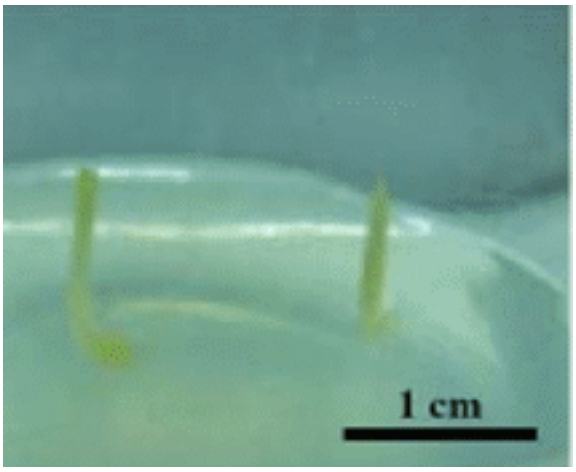

Figure 3: *In vitro* establishment of turmeric rhizome buds (Srirat *et al*., 2008)

Source and type of Explant and Sterilization

Growth of plants in a greenhouse providing desired environmental conditions that would enhance the quality of explants and will ensure the provision of planting materials throughout the year (Gurav et al., 2020). Usually it is recommended to use sprouting rhizome buds for the *in vitro* culture. If dormant buds are used, hormones should be added to induce sprouting of buds for the culture establishment (Seran 2016).

Many studies related to development of a proper sterilization procedure have been conducted worldwide. Usually the explants are pre-sterilized using running tap water to remove soil particles from planting materials (Gurav et al., 2020). According to Naz et al. (2009) the explants were surface sterilized using a detergent followed by washing with tap water. Then they were washed with 70% ethanol for 30-40 seconds. Sodium hypochlorite 20-50% was used as a sterilizer for 15 minutes and washed three times with autoclaved distilled water. Similarly, in many studies, as a pre-sterilizing agent researches have used house hold detergents, *Tween*20 with running tap water (Tyagi et al., 2004; Tyagi et al., 2007). Using Carbendazim (a fungicide) in initial sterilization would be beneficial in removal of fungal contaminations from the explants (Srirat et al., 2008). The explants are washed with 70% ethanol for 30-60 seconds (Salvi et al., 2002; Babu et al., 2016; Sandhyarani et al., 2018). The time of exposure to ethanol is reduced so as to stop bleaching of the explants. The sterilization process is then proceed to washing with 0.1% (w/v) $HgCl_2$ (mercuric chloride) solution followed by washing with sterilized distilled water. The time can be changed from 10-20 minutes to remove any traces of $HgCl_2$ (Salvi *et al*., 2002; Nasiruzzman *et al*., 2005). Sterilizing these underground rhizome buds using both the detergents and sterilizing agents is much effective on removal of bacterial and fungal contaminants. Commercially available bleaching agents like Clorox or ethanol can also be used as substituents for mercuric chloride and sodium hypochlorite. Further bacterial contamination could be minimized by adding antibiotics to the initial culture medium.

Generally explants having the size of 1-1.5 cm which consist of the shoot tip and a small portion of rhizome is used for culture (Salvi *et al*., 2002). Selecting smaller explants have lower ability of transmitting contaminants but they have higher tendency towards damaging during washing, sterilization and handling where as big sized explants are difficult to purify effectively (George *et al*., 1996). Therefore selecting explants which have the proper size to withstand the washing and handling steps as well as which have lesser tendency to transmit diseases is a must.

Culture Medium and Establishment of Explants

Plant growth and development are equally important factors which need to be considered during *in vitro*

propagation. Therefore culture medium which provides nutrients and holds the plant during *in vitro* culture is a crucial factor which must be monitored for a better regeneration of entire plant from a small explant (Thrope, 2007). MS medium is the most extensively used *in vitro* plant regeneration basal medium which consist of macro-nutrients, micro-nutrients, vitamins, minerals, carbon source and a solidifying agent as the primary constituents (Murashige & Skoog, 1962). Plant growth regulators play a vital role in development of explants in a culture medium. Auxins and cytokinins are the most commonly used growth regulators where high concentration of auxins favors root elongation while high concentration of cytokinins promotes shoot elongation. The callus formation is induced by equal amounts of auxins and cytokinins (Hussain et al., 2012). Tyagi et al. (2007) have mentioned in their study for culture establishment, combination of MS medium with BAP (6-benzylaminopurine) to ensure the growth of explants and to obtain contamination free cultures. They have further developed a low-cost culture media where commercial grade sugar was used as the sucrose supplement and Isabgol gelled media is used as the solidifying or gelling agent in place of agar. Both these compounds haven't showed any adverse effect on the explant growth and at the mean time decreases the cost of the media considerably. When it comes to incubation, the incubation environment is an important factor where conditions such as temperature, photoperiod and relative humidity should be maintained. Cultures are generally incubated under a white light for a photoperiod of 16 hours in light and 8 hours in dark per day at a relative humidity of 60-70% and a temperature of 25 ± 2^0C in an aseptic culture room (Sinchana et al., 2020).

Shoot Multiplication of Turmeric

Studies have shown that highest shoot multiplication rate was obtained when MS medium was formulated with cytokinins and auxins (Figure 4). The media were supplemented with 6-benzylaminopurine (BAP), 6-benzyladenin (BA) or Kinetin (Kn) either individually or in combination with α-naphthaleneacetic acid (NAA) or indole-acetic acid (IAA) have a significant effect on *in vitro* micro rhizome induction. Plant growth regulators in the combination of 12 µM of BA with 0.3µM of NAA gives a better response on mean number of shoots of 8.1±0.36 and weight per shoot of 0.67±0.03 g while the Kn alone or in combination with NAA doesn't give any promising results (Islam et al., 2004). According to Rahman et al. (2004) the best shoot proliferation from rhizome bud explants were observed in the culture media which contains MS+2.0 mgl^{-1} BA (6-benzyladenin) where 100% of the explants produces shoots. In addition, effective shoot regeneration was achieved when explants were treated with 2.0mg/l BAP + 0.2mg/l IAA (94.44%) followed by 2.0mg/l BAP + 0.5 mg/l IAA (86.10%) (Sinchana et al., 2020). Similar results have been observed by several other research (Keshavachandara & Khder 1989; Balachandran et al., 1990).

Figure 4: Shoot establishment in turmeric rhizome buds (Swarnathilaka & Nilantha, 2012)

In addition, evidence is available to prove that combination of 3.0mg/l BAP + 3.0mg/l IAA produces highest number of shoots (4.20) with highest shoot length (5.22cm) (Deanne et al., 2018). This was further confirmed by Singh et al. (2011) and Sahoo et al. (2011) that the combination of BAP (3.0mg/l) and IAA (1.0mg/l) was optimum for shoot initiation in *C.longa*. Furthermore, research findings of Nasiruzzaman et al., (2005) showed that using 4.0mg/l BAP with 1.0mg/l NAA in Woody Plant Medium gives 95% of plant regeneration rate within 8-10 days of inoculation. Similarly, such results were showed by Swarnathilaka & Nilantha (2012) where high shoot multiplication rate was obtained when treated with 4.0mg/l BAP + 0.50mg/l NAA in MS medium. Therefore, these results conclude that BAP concentrations of 2.0 - 4.0mg/l can be used in effective regeneration of turmeric in combination with auxins. However minimum percentage of shoot initiation would be observed when the BAP and IAA levels are decreased (Gomathy et al., 2014).

Family *Zingiberaceae* is highly responsive to BAP treatments where proper combination of growth

hormones at desired concentrations increases the shoot initiation and multiplication rate that may be due to nutrient uptake and plantlet growth because of synergic effect of plant growth regulators (Ali et al., 2009).

Root Induction

The root induction was observed from IAA, IBA and NAA, auxins when incorporated to the basal medium. Literature survey revealed that IBA is the most promising growth regulator among the three frequently used auxins (Sinchana et al., 2020). Plantlets regenerated on half MS medium with 2.00 mg/l IBA used by several researches to obtain better rooting of the turmeric. Some other studies have also revealed the better rooting ability of IBA even though they have used different concentrations. According to Rahman et al. (2004) 0.2mg/l IBA could result in 100% rooting with six weeks of culture. The highest number of shoots were produced by 5.0 mg/L IBA per shoot (Deanne et al., 2018).

However, the use of 0.01mg/L NAA also resulted in higher root number in turmeric (Suminar et al., 2018). Similarly, Shahinozzaman et al. (2013) also have found that NAA was best for the root induction in black turmeric (*C. caesia*) over IBA. High concentrations of exogenous auxins inhibit the growth rate of roots as it decreases the activity of endogenous auxin in plants where they also confirmed the increase of root number by auxins but inhibition of root elongation (Alarcon et al., 2012).

Acclimatization

The environmental conditions are maintained under the *in vitro* conditions to ensure the growth of plantlets. Hence, transferring of *in vitro* plantlets to the *ex vitro* conditions and field should be maintained carefully due to the changes in environmental conditions and also morphology of plantlets particularly in stomata and cuticle (Seran, 2016). High level of relative humidity is a major factor for a better acclimatization of *in vitro* propagated turmeric plantlets. This was confirmed in the study where it used a Sand: Soil: Peat (1:1:1) combination as the potting media which shows a successful acclimatization rate of 70%-80% under greenhouse conditions up to 30-50 days. It was also found that moderate temperature is also a crucial factor (Naz et al., 2009). The effect of relative humidity was further confirmed by Zapata et al. (2003) where they have used a hydroponic system to maintain relative humidity and to provide nutrient requirements during acclimatization under *ex vitro* conditions. Deanne et al. (2018) achieved a success rate of 80% of *in vitro* transferred plants in potted soil while no morphological abnormalities found when grown under greenhouse conditions. Further when plantlets were transferred to 1:1:1 ratio soil, cow dung and sand mixture they also showed a survival rate of 96% under field conditions after a one-month growth in green house (Nayak et al., 2011).

About 70% of the transplant *in vitro* propagated plants survive under *ex vitro* environment while other 30% could not survive either due to desiccation or necrosis occurred by microbial growth was also confirmed by a study (Rahman et al., 2004).

Conclusion

Sprouting rhizome buds are the most extensively used explant types for *in vitro* propagation of *Curcuma longa*. Sterilization procedure involves the washing of explant with ethanol followed by an antiseptic agent like Mercuric chloride or a commercial bleach like Clorox. Commonly used culture medium for explant establishment is MS medium. Shoot multiplication was successfully done when the media was supplemented alone with cytokinin (BAP, BA, Kn) or in combination with auxins (NAA, IAA) (2.0-4.0 mg/L of BAP were found as best hormone concentrations for shoot initiation). Auxins such as IAA, IBA or NAA (2.0 mg/L IBA) are used in the media for the induction of roots. Several studies have been proved that 70-90% survival rate of *in vitro* plantlets could be obtained when transferred into the field conditions where Sand: Soil: Peat (1:1:1) combination was used as the potting media.

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