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# Some Useful Information about Micropropagation

Behzad Kaviani
Department of Horticultural Sciences, Rasht Branch, Islamic Azad University, Rasht, Iran

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*Corresponding author's email: b.kaviani@yahoo.com

Abstract

This review article investigates a comprehensive description of the factors, methods, strategies, approaches and prerequisites influencing *in vitro* micropaopagation and the amount of their use across the world. The findings presented here are the results of the study of several hundred papers published all over the world. Tissue culture techniques are routinely used for micropropagation. Enhancement of efficiency and efficacy of plant regeneration are primary goals of micropropagation. In this article, maximum and minimum use of some basic issues concerned with plant tissue culture *in vitro* especially micropropagation such as types of explants, types of culture media, types of sterilizing agent for explants, types of plant growth regulators (PGRs) for general studies of miropropagation, shoot and root induction, somatic embryogenesis (SE) and callus induction have been considered. Maximal application for explants, culture media and sterilizing agent are single node, Murashige and Skoog (MS) and sodium hypochlorite (NAClO), respectively. BA and NAA are the most application among cytokinins (CKs) and auxins for general studies of miropropagation. Among all PGRs used for general studies of miropropagation, NAA is on the top. BA and IBA are the most use among CKs and auxins for shooting and rooting of explants, respectively. 2,4-D, NAA and TDZ are used more than the other PGRs for induction of SE. Among all types of auxins and CKs used as singular or in combination with them for callus induction, 2,4-D is at the top level. Combination of BA and NAA is the maximum for general studies of miropropagation. This review article can help to the future studies on micropropagation due to the correct selection of the treatments

**Keywords:** Callus, *In vitro* culture, Root induction, Shoot multiplication, Somatic embryogenesis, Tissue culture.

**Abbreviations:** BA: 6-benzyladenine, BAP: 6-benzylaminopurine, CK: cytokinin, 2,4-D: 2,4-dichlorophenoxyacetic acid, $GA_3$: gibberellic acid, IAA: indole-3-acetic acid, IBA: indole butyric acid, 2-ip: 2-isopentenyl adenine, KIN: kinetin, MS: Murashige and Skoog, NAA: α-naphthalene acetic acid, PGRs: plant growth regulators, SE: somatic embryogenesis, TDZ: thiadiazuron, ZA: zeatin.

## INTRODUCTION

Micropropagation is a technique of rapidly multiplying stock plant material to produce a large number of progeny plants, using plant tissue culture methods. Micropropagation is used to multiply plants those that have been genetically modified, bred through conventional plant breeding methods, does not produce seeds and does not respond well to vegetative reproduction. Micropropagation has become an important part of the commercial propagation of many plants because of its advantages as a multiplication system (Iliev *et al.*, 2010). Several techniques for *in vitro* plant propagation have been devised, including the induction of axillary and adventitious shoots, the culture of isolated meristems and plant regeneration by organogenesis and/or somatic embryogenesis (SE) (Iliev *et al*., 2010). In spite of all the problems, micropropagation of plants is still an efficient system and is being used by many commercial laboratories and national institutes worldwide for rapid plant multiplication, germplasm conservation, pathogen elimination, genetic manipulations and for secondary metabolite production. The great potential of micropropagation for large-scale plant multiplication is reduction of the cost by applying low-cost tissue culture (Kozai *et al.*, 1997; Altman and Loberant, 1998; Rathore *et al.*, 2004). Tissue culture has in recent years been recognized as an important tool in agricultural and horticultural development, and most popular application of this technique is micropropagation (Rathore *et al.*, 2004; Jain and Ochatt, 2010). This technique is most suitable for mass propagation and the establishment of disease free stock material. Micropropagation has improved research and development in many fields of plant science such as physiology and biotechnology. Tissue culture has allowed mass propagation of superior genotypes, thus enabling the commercialization of healthy and uniform planting material (Winkelmann *et al.*, 2006; Nhut *et al.*, 2006). Micropropagation of selected virus-free plants and elimination of viruses during *in vitro* culture are the best methods to ensure that the breeding and planting stocks are healthy (Iliev *et al.*, 2010). It is well known that the quality of plantlets cultured *in vitro* is affecting the acclimatization of micropropagated plants of many species. Rooting and acclimatization are the points to be focused in order to obtain protocols suitable for massive commercial systems. Techniques of micropropagation and production of disease free plant stocks have been defined and refined to such an extent that they have become standard practice for a range of crop plants. Plant tissue culture can be considered to involve three phases. First, isolation of the plant (tissue) from its usual environment. Second, the use of aseptic techniques to obtain material free of bacterial, fungal, viral and even algal contaminations. Third, the culture and maintenance *in vitro* in a strictly controlled physical and chemical environment (Kozai *et al.*, 1997; Nguyen and Kozai, 1998; Smith, 2000). The success of this technology is to a great extent dependent upon abiding by a number of fundamental rules and following a number of basic protocols. Some protocols, at least at the moment, are more efficient than others. General rules are sometimes difficult to make because of the variability of response to particular protocols of different plant species or even cultivars. Knowledge of the optimal conditions favoring differentiation under *in vitro* conditions is an important step in the application of *in vitro* manipulation techniques in plants (Gao *et al.,* 2010). Successful plant regeneration depends on factors such as the genotype, explant type, age of the donor plants, the number of subcultures (Jain, 1998; Veilleux and Johnson, 1998), and the composition of medium, especially PGRs. Here, this review article evaluates affecting factors on micropropagation such as types of explants, types of culture media, types of sterilizing agent for explants, types of PGRs for general studies of miropropagation, shoot and root induction, SE and callus induction and the percentage of their use across the world in order to the better decision for experimental works.

### Types of explants

Explant culture is a technique used for the isolation of cells from a pieces of tissue. Tissue obtained from the plant to culture in this procedure is called an explant. It can be a portion of the shoot, leaves, or some cells from a plant. Selection of explant is very important for tissue culture

Table 1. Types of explants and the percentage of their use across the world.

| Type of explant | Single node (axillary bud) | Leaf | Shoot tip (apical bud) | Other parts of plants* |
|---|---|---|---|---|
| The percentage of the use | 40.60 | 18.81 | 16.83 | 23.76 |

*Such as petiole, root, internode, inflorescence, floral parts, rhizome, hypocotyl, cotyledon, seed, embryo, embryonic axes, bulb and fruit.

process and must be selected correctly. In many species, explants of various organs vary in their rates of growth and regeneration. The risk of microbial contamination is increased with inappropriate explants. Thus, the choice of appropriate explant is very important prior to tissue culture. Some important factors consist differences in the stage of the cells in the cell cycle, the ability to transport endogenous PGRs and the metabolic capabilities of the cells (Iliev *et al.*, 2010; Jain and Ochatt, 2010). The most commonly used tissue explants are the meristematic ends of the plants such as node and the shoot tip (apical and axillary buds). This review article showed that the single node (axillary bud) explants are extremely used across the world (Table 1). Also, leaves are good explants especially for callus induction due to the presence of meristem and parenchyma cells (Table 1). These tissues have high rates of cell division and either concentrate or produce required growth regulating substances including auxins and CKs. Elimination of microbes from aerial parts explants is easier than roots. An alternative for obtaining uncontaminated explants is to take explants from seedlings grown from surface sterilized seeds under aseptic conditions. The hard surface of the seeds is less permeable to surface sterilizing agents, so the acceptable conditions of sterilization used for seeds may be more stringent than for vegetative tissues. Many characters of the explant are known to affect the efficiency of culture initiation. Generally, younger, more rapidly growing tissue (or tissue at an early stage of development) is most effective. Overall, plant cell culture encompass all aspects of the cultivation and maintenance of plant material *in vitro.*

**Types of culture media**

A growth medium or culture medium is a liquid or gel designed to support the growth of explants obtained from plants and microorganisms (Madigan and Martinko, 2005). There are different types of media for growing different types of cells. The basic protocols were well established by the 1960s and a whole research field and industry grew based on the ubiquitous MS (Murashige and Skoog, 1962) medium. The composition of the culture medium depends upon the plant species, the explants, and the aim of the experiments. In general, certain standard media are used for most plants, but some modifications may be required to achieve genotype-specific and stage-dependent optimizations, by manipulating the concentrations of growth regulators, or by the addition of specific components to the culture medium. The large number of media have been presented by researchers. Among these culture media, MS medium is used most extensively (Table 2).

**Types of sterilizing agent for explants**

Plant tissue culture is performed under aseptic conditions under filtered air. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of explants in chemical solutions is required. Mercuric chloride ($HgCl_2$) is seldom used as a plant sterilizing agent today, as it is dangerous to

Table 2. Types of culture medium and the percentage of their use across the world.

| Type of culture medium | MS | Other media* |
|---|---|---|
| The percentage of the use | 96.15 | 3.75 |

*Such as Knudson, Knop, Chu (N6), Gamborg (B5), Greshoff and Doy (GD), Linsmaier and Skoog (LS), Shenck and Hildebrandt (SH), Nitsch and Nitsch and Heller.

Table 3. Types of sterilizing agent and the percentage of their use across the world.

| Type of sterilizing agent | NaClO* | $HgCl_2$ | Other sterilizing agent** |
|---|---|---|---|
| The percentage of the use | 68.88 | 26.41 | 3.00 |

* In 95% of the cases, NaClO is used accompanying with ethanol 70%. In most cases, a surfactant (wetting agent) is an accompanying, too, **Such as $H_2O_2$, calcium hypochlorite, alcohol, antibiotics and bromine water

Table 4. Types of CKs used for general studies of miropropagation and the percentage of their use across the world.

| Type of CK | BA | BAP | TDZ | KIN | 2-iP | ZA |
|---|---|---|---|---|---|---|
| The percentage of the use | 41.10 | 21.91 | 15.06 | 12.32 | 8.22 | 1.36 |

use and is difficult to dispose. The disinfection of explants before culture is essential to remove surface contaminants such as bacterial and fungal spores. Surface disinfection must be efficient to remove contaminants, with minimal damage to plant cells. Sodium hypochlorite is a chemical compound with the formula NaClO. It is composed of a sodium cation ($Na^+$) and a hypochlorite anion ($ClO^-$). It may also be viewed as the sodium salt of hypochlorous acid. When dissolved in water it is commonly known as bleach or liquid bleach, and is frequently used as a disinfectant or a bleaching agent. NaClO, especially in combination with ethanol is used maximal across the world (Table 3). This article revealed that the use of $HgCl_2$ is more than the other sterilant, except for NaClO, in spite of its disadvantages.

**Types of CKs used for general studies of micropropagation**

CKs are a class of PGRs that promote cell division in plant roots and shoots. They are involved primarily in cell growth and differentiation and axillary bud growth in plant tissue culture. There are two types of CKs: adenine-type CKs represented by KIN, ZA and BAP, and phenylurea-type CKs like diphenylurea and TDZ. CKs participate in local and long-distance signalling, with the same transport mechanism as purines and nucleosides (Sakakibara, 2006). CKs act in concert with auxin, having generally opposite effects. The ratio of auxin to CK plays an important role in the effect of CK on plant growth. CK alone has no effect on parenchyma cells. When cultured with auxin but no CK, they grow large but do not divide. When CK is added, the cells expand and differentiate. More CK induces growth of shoot buds. CKs are involved in many plant processes, including cell division and shoot and root morphogenesis. They are known to regulate axillary bud growth and apical dominance. As I expected, among all CKs, BA is used most extensively (Table 4). There is no significant difference between the use of adenine-type CKs and phenylurea-type CKs across the world (Table 4).

**Types of auxins used for general studies of micropropagation**

Auxins are a class of PGRs with some morphogen-like characteristics. Auxins have a cardinal role in coordination of many growth and behavioral processes in the plant's life cycle and are essential for plant body development. The pattern of auxin distribution within the plant is a key factor for elongation during growth of the stem and root, its reaction to its environment, and specifically for development of plant organs (such as leaves or flowers) (Friml, 2003). Auxins typically act in concert with, or in opposition to, other PGRs. For example, the ratio of auxin to CK in certain plant tissues determines initiation of root versus shoot buds. The most important member of the auxin family is IAA. The four naturally occurring (endogenous) auxins are IAA, 4-CIAA, PAA and IBA. Synthetic auxins analogs include NAA, 2,4-D and many others. Auxins are also often used to promote initiation of adventitious roots. On the cellular level, auxin is essential for cell growth, affecting both cell division and cellular expansion. Auxin concentration level, together

Table 5. Types of auxins used for general studies of micropropagation and the percentage of their use across the world.

| Type of auxin | NAA | IAA | IBA | 2,4-D |
|---|---|---|---|---|
| The percentage of the use | 70.58 | 17.64 | 7.84 | 3.92 |

Table 6. Types of PGRs used for general studies of micropropagation and the percentage of their use across the world.

| Type of PGRs | NAA | BA | BAP | TDZ | KIN | IAA | 2-iP | GA3 | 2,4-D | ZA |
|---|---|---|---|---|---|---|---|---|---|---|
| The percentage of the use | 28.12 | 23.44 | 12.50 | 8.60 | 7.03 | 7.03 | 4.68 | 2.34 | 1.56 | 0.78 |

with other local factors, contributes to cell differentiation and specification of the cell fate. Auxin stimulates cell elongation by stimulating wall-loosening factors, such as elastins, to loosen cell walls. The effect is stronger if gibberellins are also present. Auxin also stimulates cell division if CKs are present. When auxin and CK are applied to callus, rooting can be generated if the auxin concentration is higher than CK concentration. Auxin also induces sugar and mineral accumulation at the site of application. Auxin induces both growth of pre-existing roots and adventitious root formation, i.e., branching of the roots. High concentrations of auxin inhibit root elongation and instead enhance adventitious root formation. Auxins are toxic to plants in large concentrations. Used in high doses, auxin stimulates the production of ethylene. As I expected, the use of NAA is highest among all auxins across the world (Table 5). There is significant difference between the use of NAA and other auxins. 2,4-D is a potent synthetic auxin and uses for some other goals like callus induction and SE induction.

**Types of PGRs used for general studies of micropropagation**

The composition of the medium, particularly PGRs have found effects on the growth and differentiation of the tissues. There is some considerable difficulty in predicting the effects of PGRs: this is because of the great differences in culture response among species, cultivars, and even plants of the same cultivar grown under different conditions. Auxins and CKs are the most widely used PGRs in plant tissue culture and are usually used together, the ratio of the auxin to the CK determining the type of culture established or regenerated (Peeters *et al.,* 1991). A high auxin to CK ratio generally favors root formation, whereas a high CK to auxin ratio favors shoot formation. An intermediate ratio favors callus production. For successful plant micropropagation, the crucial requirements for an auxin and CK are well documented in the botanical literature. I expected that BA is used in the highest rate among all PGRs, but after this research, I found that NAA is first in use. BA is at the second level (Table 6). Reasons for this are as follows: first; rooting is a crucial and harder step in micropropagation which stimulate by auxins especially NAA, second; NAA at low concentrations is a good accompanying hormones with CKs like BA for shoot multiplication, third; NAA is used maximal for general studies of miropropagation among other auxins.

**Types of PGRs used for shooting**

The success of the micropropagation method depends on several factors like genotype, media, PGRs and type of explants, which should be observed during the process (Pati *et al.*, 2005; Nhut *et al.*, 2010). Most important of these parameters are PGRs included in the culture media (Gomes and Canhoto, 2003). PGRs act like signals to stimulate, inhibit or regulate growth in the developmental programs of plants (Mercier *et al.*, 1997). CKs are usually used on the micropropagation media to stimulate axillary shoot proliferation (Chawla, 2009; El-Agamy, 2009). However, the ideal concentrations differ from species to species and need to be established accurately to obtain the effective rates of multiplication. In general, three modes of *in vitro* plant regeneration have been in practice: organogenesis, embryogenesis and axillary proliferation. In tissue culture, CKs

Table 7. Types of PGRs used for shooting in micropropagation and the percentage of their use across the world.

| Type of PGRs | BA | NAA | BAP | KIN | TDZ | $GA_3$ | IAA | IBA | ZA | 2-iP | 2,4-D |
|---|---|---|---|---|---|---|---|---|---|---|---|
| The percentage of the use | 24.60 | 20.31 | 14.45 | 8.98 | 8.20 | 6.25 | 5.07 | 3.90 | 3.51 | 2.73 | 1.95 |

Table 8. Types of PGRs used for rooting in micropropagation and the percentage of their use across the world.

| Type of PGRs | IBA | NAA | IAA | 2,4-D | BAP | BA | KIN | Other PGRs |
|---|---|---|---|---|---|---|---|---|
| The percentage of the use | 45. 32 | 30.10 | 12.66 | 5.50 | 2.10 | 2.10 | 1.11 | 1.11 |

and auxins play a crucial role as promoters of cell division and act in the induction and development of meristematic centers leading to the formation of organs (Peeters *et al.*, 1991). CKs are usually used on the micropropagation media to stimulate axillary shoot proliferation. Many researchers showed that CK BA induced multiple shoot formation and shoot length (Lin *et al*., 1997; Nhut, 2003; Fráguas *et al.*, 2004; Raj Poudel *et al.,* 2005; Hashemabadi and Kaviani, 2008, 2010; Kaviani 2014). This article confirmed this finding. BA is used maximum among all PGRs for shoot induction (Table 7). At the stage of shoot proliferation and multiple shoot production, each explant has expanded into a cluster of small shoots. Multiple shoots are separated and transplanted to new culture medium (Iliev *et al*., 2010). Shoots are subcultured every 2–8 weeks. Material may be subcultured several times to new medium to maximize the quantity of shoots produced. Adventitious shoot formation is one of the plant regeneration pathways *in vitro*, and is employed extensively in plant biotechnology for micropropagation and genetic transformation, as well as for studying plant development (Iliev *et al.,* 2010). CKs are often applied to stem, shoot or leaf cuttings to promote adventitious bud and shoot formation (Iliev *et al*., 2010). Somaclonal variation, which may be useful or detrimental, may occur during adventitious shoot regeneration (Iliev *et al.*, 2010). In spite of the more important role of CKs on shooting, I found that the use of auxin NAA for shooting is higher than all CKs except for BA (Table 7).

**Types of PGRs used for rooting**

Rooting is a crucial step to the success of micropropagation. Auxins enhance the germination, root induction and seedling growth of many species (Jain and Ochatt, 2010; Kaviani *et al.,* 2011; Kaviani and Zakizadeh, 2013). The rooting stage prepares the regenerated plants for transferring from *in vitro* to *ex vitro* conditions in controlled environment rooms, in the glasshouse and, later, to their ultimate location (Iliev *et al.,* 2010). This stage may involve not only rooting of shoots, but also conditioning of the plants to increase their potential for acclimatization and survival during transplanting. The induction of adventitious roots may be achieved either in vitro or ex vitro in the presence of auxins (Iliev *et al.*, 2010). I surprised when I found that the application of IBA is higher than NAA for rooting (Table 8). Contrary to shooting that the use of auxins is high, all auxins are applied more than all CKs for rooting across the world (Table 8).

**Types of auxins used for rooting**

For any micropropagation protocol, successful rooting of microshoots is an important prerequisite to facilitate their establishment in soil. Auxins enhance the germination, root induction and seedling growth of many species (Jain and Ochatt, 2010; Zakizadeh *et al.,* 2013; Ghaffari Eisizad *et al*., 2012). Pierik (1987) indicated that NAA is a strong auxin and relatively low concentrations are needed for root formation. With high concentrations of NAA, root formation fails to occur and callus formation takes place. I think that is why IBA has been used some more than NAA for rooting across the world (Table 9). Microshoots can produce some auxins for root induction, thus, I found that 5.12% of microshoots produce roots without any PGRs (Table 9). *In vitro* shoot proliferation and multiplication are largely based on media formulations including CKs as

Table 9. Types of auxins used for rooting and the percentage of their use across the world.

| Type of auxin | IBA | NAA | IAA | Without hormones |
|---|---|---|---|---|
| The percentage of the use | 41.02 | 33.33 | 20.51 | 5.12 |

Table 10. Types of PGRs in combination with them used for rooting in micropropagation and the percentage of their use across the world*.

| Type of PGRs | NAA + BA | NAA + KIN | NAA + TDZ | NAA + IBA | IBA + KIN | IAA + BA | IAA + IBA |
|---|---|---|---|---|---|---|---|
| The percentage of the use | 36.36 | 18.18 | 9.10 | 9.10 | 9.10 | 9.10 | 9.10 |

*In 78% of the cases, single hormone and in 21% of the cases, two hormones in combination with them have been applied for rooting

Table 11. Types of PGRs as singular or in combination with them used for somatic embryogenesis and the percentage of their use across the world.

| Type of PGRs | 2,4-D | NAA | TDZ | BA + NAA | BA + IAA | IAA | IAA + BAP | BA + Picloram |
|---|---|---|---|---|---|---|---|---|
| **The percentage of the use** | 13.04 | 13.04 | 13.04 | 8.70 | 8.70 | 4.34 | 4.34 | 4.34 |
| **Type of PGRs** | **NAA + BAP** | **KIN** | **BAP** | **IBA** | **IAA + NAA** | **NAA + 2,4-D** | **BA + 2,4-D** | - |
| **The percentage of the use** | 4.34 | 4.34 | 4.34 | 4.34 | 4.34 | 4.34 | 4.34 | - |

a major PGR, while, in some cases, low concentrations of auxins and $GA_3$ were also used (Pati *et al.*, 2005).

## Types of PGRs used for rooting

Rooting is an important process to the success of micropropagation. Without effective root system plant acclimatization will be difficult and the rate of plant propagation may be severely affected (Gonçalves et al., 1998). Auxin type and concentration significantly influenced rooting percentage and root length. As I expected, the use of NAA along with BA is the highest among all other combination of auxins and CKs across the world (Table 10). There is significant difference between the uses of NAA in combination with BA with other combinations of PGRs (Table 10).

## Types of PGRs used for SE

SE was defined by Emons (1994) as the development from somatic cells of structures that follow a histodifferentiation pattern which leads to a body pattern resembling that of zygotic embryos (Iliev *et al.*, 2010). This process can be induced *in vitro* in some species. SE may occur directly from cells or organized tissues in explants or indirectly through an intermediate callus stage. It has been confirmed in many species that the auxins 2,4-D and NAA, in the correct concentrations, play a key role in the induction of SE. Current research confirm this statement. Of course, I understood that TDZ, as a CK also is applied as same as 2,4-D and NAA across the world (Table 11). In some species (such as *Abies alba*) CKs on their own induce SE (Iliev *et al.,* 2010). The present review showed that in some cases, combination of an auxin with a CK can be useful for induction of SE (Table 11). Application of the CKs, BAP or KIN, may enhance plant regeneration from somatic embryos after the callus or somatic embryos have been induced by auxin treatment (Jain and Ochatt, 2010; Kaviani, 2013). The induction of direct SE for in vitro plant regeneration provides several advantages over the traditional organogenesis (Wang and Bhalla, 2004). SE provides an excellent morphogenetic system for investigating the cellular and molecular process underlying differentiation (Benelli *et al.*, 2001). In addition, SE also provides the possibility to produce artificial seeds and valuable tools for genetic engineering and germplasm conservation and exchange by cryopreservation (Litz and Gray, 1995; Merkle, 1997). SE is a model system for studying the genetic basis of embryogenesis and totipotency of somatic cells (Ewa *et al.*, 2007). Additionally, direct SE has a lower probability of genetic variation than other propagation methods

Table 12. Types of PGRs as singular or in combination with them used for callus induction and the percentage of their use across the world.

| Type of PGRs | 2,4-D | 2,4-D + BA | 2,4-D + BAP | NAA + BA | NAA | BAP + NAA | 2,4-D + KIN |
|---|---|---|---|---|---|---|---|
| The percentage of the use | 36.08 | 10.30 | 10.30 | 7.21 | 7.21 | 4.12 | 4.12 |
| Type of PGRs | 2,4-D + TDZ | TDZ | BAP | KIN | BA + Picloram | IBA + TDZ | IBA + BAP |
| The percentage of the use | 3.09 | 3.09 | 2.06 | 2.06 | 1.03 | 1.03 | 1.03 |
| Type of PGRs | ZA | 2,4-D + NAA + KIN | 2,4-D + BAP + 2-iP | BA | IBA | 2,4-D + ZA | NAA + TDZ |
| The percentage of the use | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 | 1.03 |

(Merkle, 1997; Balaraju *et al.,* 2011; Kothari *et al*., 2010). Induction of somatic embryos directly from plant tissue is the most desirable approach because it appears to be associated with the cytological and genetic stability of regenerated plantlets (Vasil, 1988; Pedroso and Pais, 1995). SE acts as a key component of *in vitro* propagation. SE provides a precious implement to boost the pace of genetic enhancement of commercial crop species (Stasolla and Yeung, 2003).

**Types of PGRs used for callus induction**

Callus is defined as an unorganized tissue mass growing on solid substrate. Usually the first step in plant tissue culture is callus induction. Callus is an amorphous tissue consisting of dedifferentiated, unorganized cell masses (George *et al.*, 2008). Callus formation is central to many investigative and applied tissue culture procedures (biotechnology) (Mineo, 1990). Callus is a good source for suspension culture, production of secondary metabolites, clonal propagation through SE and organogenesis, also, the study of cell division, elongation and differentiation process and genetic transformation (Mineo, 1990). Among all culture methods, cell suspension culture (obtained via callus) represents the best system for producing secondary metabolites because of their fast growth rate (Chan *et al*., 2010; Praveen and Murthy, 2011). Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies (Mineo, 1990). PGRs have important role in callus induction and growth. 2,4-D has the most application for this purpose (Jain and Häggman, 2007; Ahmadi Hesar *et al*., 2011). This review article confirms this statement. Explants from several parts of intact plants can be used to form callus. The most successful explants are often young tissues of one or a few cell types (Mineo, 1990). Explants, when cultured on the appropriate medium, usually with both an auxin and a CK, can give rise to callus. Callus culture is often performed in the dark as light can encourage differentiation of the callus (Khorrami Raad *et al.*, 2012). During long term culture, the culture may lose the requirement for auxin and/or CK. Callus cells proliferate without differentiating, but eventually differentiation occurs within the tissue mass. The extent of overall differentiation usually depends on the hormone balance of the support medium and the physiological state of the tissue. Manipulation of the auxin to CK ratio in the medium can lead to the development of shoots, roots, or somatic embryos from which whole plants can subsequently be produced. A typical plant callus will undergo three stages of development (George *et al.*, 2008). The first stage is the induction of cell division. The second stage is dedifferentiation, which is a period of active cell division during which differentiated cells of the explants lose any specialized feature. Finally the last stage is the period during which cell division decreases or ceases and cellular differentiation increases within the callus (George *et al*., 2008). Many parameters like explants, species, cultivar, PGRs and light affect callus induction, callus growth and callus quality for regeneration. Among PGRs, auxins and CKs are widely used for callus induction. Auxins and CKs seem to be necessary for plant cell division. In plant tissue culture, auxins are broadly used for callus induction. Auxins are involved in cell division, cell elongation, vascular tissue differentiation, rhizogenesis and root formation, embryogenesis and inhibition of axillary shoot growth (Chawla, 2002; George *et al.*, 2008; Park *et al.*, 2010). The auxin commonly used for callus induction is 2, 4-D, but NAA and IAA are also used (George *et al.*, 2008). This review article showed that it is correct for 2,4-D and NAA but not for IAA (Table 12). My researches revealed that the most PGRs applied singularly for callus induction is 2,4-D followed

Table 13. The combination of PGRs used for shooting and the percentage of their use across the world*.

| CKs \ Auxins | NAA | IAA | IBA | 2,4-D |
|---|---|---|---|---|
| **BA** | 81.25 | 9.37 | 9.37 | 00.00 |
| **BAP** | 77.77 | 22.33 | 00.00 | 00.00 |
| **TDZ** | 100.00 | 00.00 | 00.00 | 00.00 |
| **KIN** | 71.42 | 14.28 | 14.28 | 00.00 |
| **2-iP** | 57.14 | 28.57 | 00.00 | 14.28 |
| **ZA** | 100.00 | 00.00 | 00.00 | 00.00 |

*Combinations are based on the Latin Square and numbers have been presented to percentage.

by NAA. But, the use of 2,4-D is about 5 times more than NAA across the world (Table 12). Callus tissues in which cells divide in media without CKs, are able to produce their own natural CKs (George *et al.*, 2008). In relation to the combination of auxins and CKs for callus induction, the use of 2,4-D along with BA and BAP is highest among all other PGRs combinations across the world (Table 12).

**The combination of PGRs used for shooting**

In many micropropagation studies a high number of treatments especially PGRs and their dosages are tested in an effort to find the best way to obtain a good propagation protocol. Some species may require a low concentration of auxin in combination with high levels of CKs to increase shoot proliferation (Van Staden *et al.*, 2008; Kaviani *et al.*, 2013; Kaviani, 2014; Baker *et al.,* 2014). The present investigation revealed that the most frequently used PGRs for micropropagation of plants by organogenesis, embryogenesis and axillary proliferation are NAA along with BA (Table 13). The combination of 2,4-D with CKs is used the least for shooting across the world (Table 13). KIN has been applied for micropropagation of many plants (Jain and Ochatt, 2010). CKs are usually used on the micropropagation media to stimulate axillary shoot proliferation (Chawla, 2009). However, the ideal concentrations differ from species to species and need to be established accurately to obtain the effective rates of multiplication. Generally, CKs used in plant tissue culture are BA, BAP, ZA, KIN and more recently, TDZ.

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