Title: Somaclonal variation: Basic and practical aspects

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Preface

In vitro culture includes a wide set of different techniques such as micropropagation, somatic hybridization, haploid and polyploid production, *in vitro* fertilization, embryo rescue, *in vitro* mutagenesis, variant selection, or plant regeneration through organogenesis or somatic embryogenesis. These procedures can be applied to different fields with different purposes such as clonal mass propagation, virus eradication, germplasm conservation, production of secondary metabolites, basic investigations or plant breeding by using conventional means or biotechnological tools, such as genetic transformation or gene editing.

Cell and tissue culture only involve mitotic divisions of cells and, therefore, one of the main goals of *in vitro* culture is the propagation of genetically uniform plants. However, from the beginnings of *in vitro* culture, different abnormalities have been observed in the obtained plants. In 1981, Larkin and Scowcroft stated that *in vitro* culture itself is a source of variation and proposed the term "somaclonal variation" to refer to variations induced by *in vitro* culture in cells, cultures, regenerated plants and their progenies. Occurrence of this spontaneous and uncontrolled variation can represent a serious problem in most systems. However, it constitutes an alternative source of variability that can be exploited in plant breeding.

There is substantial amount of investigation on somaclonal variation. In this book, basic and practical aspects of somaclonal variation are included. Specifically, the first chapters are focused on the genetic and epigenetic basis of somaclonal variation, as well as on the main factors affecting the appearance of somaclonal variation. The second part addresses different approaches for somaclonal variation evaluation. The last chapters analyze somaclonal variation in important groups of plants or crop species, examining different aspects of somaclonal variation in these specific cases.

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Factors Affecting Tissue Culture-Induced Variations

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Abstract

In vitro culture can induce somaclonal variation, giving rise to the appearance of alterations in the cultured cells and tissues, as well as in the regenerated plants and their progenies. A wide range of alterations can emerge as a consequence of somaclonal variation, which can be detected at different levels, such as phenotypic, cytogenetic, biochemical or molecular. Both nature and frequency of changes are uncontrolled due to the unpredictable nature of somaclonal variation. Thus, somaclonal variation is considered an important drawback intrinsically associated to the use of *in vitro* culture techniques and, in fact, it constitutes a very important problem in culture systems in which true-to-typeness is expected. Nevertheless, it can also be used as a source of genetic variability which can be exploited in plant improvement. Comprehensive knowledge of the effect of different factors affecting somaclonal variation, such as the genotype, explant source, culture conditions, culture age or the *in vitro* culture method, can help to design strategies in order to reduce or promote somaclonal variation, depending on the objectives that have determined the use of *in vitro* culture.

Keywords

Culture age, explant source, genotype, *in vitro* culture, plant growth regulator, somaclonal variation

1 Introduction

In vitro culture is at the base of different techniques with multiple applications in different fields such as commercial plant production, basic research or plant breeding by conventional or biotechnological means. During *in vitro* culture, cells and tissues are exposed to different stresses, from explant excision to the artificial conditions on which they are incubated, which may induce genetic and epigenetic changes (Neelakandan and Wang 2012).

The term ‘somaclonal variation’ was coined by Larkin and Scowcroft (1981) to refer to alterations induced by *in vitro* culture in the cell, tissues, regenerated plants and their progenies. Somaclonal variation (SV) occurs in a spontaneous, random and uncontrolled way (Karp 1994) and, therefore, it is unavoidable and unpredictable in nature (Jain 2001). Very variable rates of SV have been reported depending on the culture

system, with cases in which the genetic integrity is maintained versus systems in which a high proportion of *in vitro* culture-derived plants exhibited variations.

The occurrence of SV constitutes an important problem in commercial clonal propagation, germplasm conservation and in genetic transformation or gene editing experiments, in which no alterations due to tissue culture are expected. However, SV can be used as an alternative source of genetic variability in breeding programs. This option is especially interesting when other breeding methods are limited (Vitamvas et al. 2019), as occurs in trees and long-lived perennial species, vegetatively propagated plants and crops with polygenic traits or having a narrow genetic base (DeVerno 1995; Brar and Jain 1998).

Due to the importance of SV and its consequences on multiple systems in which *in vitro* culture is used as the main technology or as a complementary tool, it is very important to know factors affecting its appearance. The comprehensive understanding of the factors influencing SV can help to design different strategies to increase or reduce its incidence, depending on the objectives of the *in vitro* culture system.

Although there is no effective method known to control this phenomenon (Bhojwani and Razdan 1996), it is well established that some factors affect the occurrence of SV and the frequency of changes. Somaclonal variations can be generated during *in vitro* culture or can be due to pre-existing variability in the tissues used as explant (Peschke and Phillips 1992). In this chapter, different factors affecting variations induced by *in vitro* culture, such the composition of the culture medium, *in vitro* culture method, starting explant, culture age, or the genotype of the mother plant, will be studied.

2. Culture conditions

Plant stability is affected by the conditions to which cells and tissues are exposed during *in vitro* culture.

2.1 Culture medium

Medium composition is a key factor influencing SV. Although plant growth regulators (PGRs) play a main role in the occurrence of SV, other components also contribute to the occurrence of SV.

In *Agave angustifolia*, nutrient limitation altered the phenotype in some genotypes, and increased DNA methylation (Duarte-Aké and De-la-Peña 2021). However, in maize, hypomethylation was reported under nitrogen and phosphorus deficiency (Mager and Ludewig 2018). The concentration of ammonium nitrate affected SV in *Senecio cruentus*, with higher frequency of variation found in shoot cultures growing on proliferation medium devoiding ammonium nitrate (Sivanesan and Jeong 2012).

2.1.1 Plant growth regulators

Exogenously applied PGRs play an essential role in tissue culture as they control morphogenesis *in vitro*. In fact, optimal concentrations and precise combinations of hormones are required for efficient micropropagation and induction of specific regeneration pathways.

Although different investigations reported no effect of PGRs on SV (Bennici et al. 2004; Venkatachalam et al. 2007), they are known to be one of the causes of SV induction. In fact, several frequently used PGRs, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthalene acetic acid (NAA), and 6-benzylaminopurine (BAP), are considered as the most prominent factors causing genetic variability (Sales and Butardo 2014).

Different works have investigated the effect of different PGRs and their concentrations on SV. SV especially increases when auxins and cytokinins were added to the culture medium (Liberatore et al. 2020). In embryogenic suspension cultures of cucumber, greater variation was found in auxin (2,4-D)-containing medium compared to that observed in cytokinin-containing medium (Ładyżyński et al. 2002). Similarly, in PLBs of Dendrobium Sabin Blue, different SV level was observed depending on the PGR. In this case, kinetin gave rise to the highest variation by both Inter Simple Sequence Repeat (ISSR) and DAMD analysis compared to NAA and thidiazuron (TDZ) (Chin et al. 2019).

Hormone combinations can also influence the occurrence of SV. Thus, incidence of mantled flowering in oil palm depended on auxin/cytokinin balance, with a relatively high auxin/cytokinin ratio resulting in the lowest incidence of variation, while a low auxin/cytokinins ratio gave rise to a high incidence of this alteration (Eeuwens et al. 2002).

Important differences have also been reported among hormones of the same group. In rye mature embryo culture, high level of auxins caused a reduction in the genomic template stability (GTS) value using Random Amplified Polymorphic DNA (RAPD) analysis, with 98.4% in calli maintained on MS medium with 2 mg l^{-1} dicamba versus 81.7% in those cultured on MS medium with 12 mg l^{-1} picloram. Moreover, DNA hypermethylation was observed at high concentrations of 2,4-D and picloram, whereas DNA hypomethylation was observed with dicamba supplementation (Aydin et al. 2016). In caladium, leaf-colour variants differed more with auxin type than with different concentrations of a specific auxin. Using equal molar concentration of different auxins, the number of variants was higher on media containing 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) or 2,4-D than in those supplemented with NAA or IBA (Ahmed et al. 2004).

Some auxins, as 2,4-D are especially mutagenic. During *in vitro* culture of oil palm zygotic embryos, 2,4-D supplementation gave rise to significant variations (Giorgetti et al. 2011). Using high 2,4-D concentrations also caused of SV in strawberry (Nehra et al. 1992), soybean (Gesteira et al. 2008) and cotton (Jin et al. 2008).

In relation to NAA, its effect in PLBs of Dendrobium Sabin Blue orchid (Chin et al. 2019) and in Caladium (Ahmed et al. 2004) were relatively low compared to other auxins, such as indole-3-acetic acid (IAA), 2,4-D and 2,4,5-T. However, an increase in SV was observed in *Galanthus transcaucasicus* when higher concentrations of NAA were incorporated in the culture medium (Asadi et al. 2021).

In relation to cytokinins, although Reuveni et al. (1993) stated that high levels of cytokinins did not directly affect SV in 'Cavendish' bananas, and Duarte-Aké and de-la-Peña (2021)) suggested that cytokinin could help to reduce variation, different effects have been observed when cytokinins were added to the culture medium. Thus, high concentrations of BAP and kinetin in cultures induced genetic variability in banana (Trujillo and de García 1996). In PLBs of Dendrobium Sabin Blue, kinetin gave rise to the highest percentage of polymorphism by both ISSR and Amplification of Minisatellite DNA (DAMD) band profiling compared to the other PGRs, like NAA and TDZ. In *Ananas comosus*, high concentration of BAP (4.0 mg l^{-1}) alone or combined with indolebutyric acid (IBA) produced dwarf variants (100%) (Halim et al. 2018). Similarly, genetic variability greatly increased in rice callus cultures incubated in high levels of BAP (30 mg l^{-1}), compared to lower concentrations (2 mg l^{-1}) (Oono 1985). Bairu et al. (2006) also reported a high rate of SV in 'Cavendish' banana (Musa AAA, cv. 'Zelig') cultured with high BAP concentration, and

in *Solanum melongena* L., the addition of BAP to the culture medium induced a high rate of polymorphism in adventitious shoots (Mançano et al. 2019). TDZ was used in shoot cultures of Dendrobium Second Love hybrid and SV did not appear after six subculture cycles (Ferreira et al. 2006). However, regenerated Indian soybean showed morphological and agronomic variations when it was cultured in TDZ-supplemented medium (Radhakrishnan and Kumari 2008).

Different causes may explain the effects of PGRs on SV. Auxins and cytokinins may affect the cell cycle (Halim et al. 2018). Therefore, while speeding up the cell cycle for proliferation, PGRs in the culture medium can disrupt it. Moreover, cell cycle involves duplication of genetic materials. Thus, application of PGRs increases the risk of genetic alterations and can affect the chromosome number and ploidy levels (Smulders and de Klerk 2011). Modulation of DNA methylation by PGRs has also been reported (Us-Camas et al. 2014; Campos-Rivero et al. 2017). Additionally, as pointed out by Pastelín Solano et al. (2019), an endogenous accumulation of PGRs in tissues probably occurs throughout subcultures, causing the excess regulators to act as a mutagenic agent.

In relation to auxins, genetic and epigenetic modifications have been found due to this PGR. In general, increased auxin concentration, especially 2,4-D and picloram led to a decrease in the GTS value, and epigenetic variations have also been observed as a consequence of auxin treatments (Aydin et al. 2016). However, while DNA hypermethylation occurred at higher concentrations of 2,4-D, picloram, and NAA, DNA hypomethylation was observed in dicamba (Loschiavo et al. 1989; Aydin et al. 2016). The presence of auxins can also cause chromosomic aberrations (Bairu et al. 2011; Mançano et al. 2019).

The synthetic auxin 2,4-D, widely used in tissue culture, has a proven mutagenic effect (Bhojwani and Dantu 2013). Thus, 2,4-D has been associated with occurrence of genetic changes (Ładyżyński et al. 2002; Mohanty et al. 2008) and promotion of DNA methylation, causing epigenetic variations (Baklouti et al. 2022). Additionally, it has been reported that the presence of 2,4-D in the culture media significantly decreased the mitotic index and increased the ploidy level in cells (Saleh et al. 2019). Nevertheless, Aydin et al. (2016) pointed that the reason behind this variation may be the stress inherent in cellular deprogramming induced by 2,4-D. Dicamba has also been considered a mutagenic agent (Aydin et al. 2016). However, substitution of 2,4-D by NAA or IAA reduced chromosomal aberrations (Bhojwani and Dantu 2013).

The use of cytokinins has also been found to increase the occurrence of polyploid cells (Liberatore et al. 2020). Specifically, BAP has been associated with epigenetic aberrations (Smulders and de Klerk 2011), cell-cycle disturbance (Bairu et al. 2011), and increase in chromosome number (Giménez et al. 2001). Moreover, in *Malus xiaojinensis*, DNA methylation decreases with increasing concentrations of BAP in the culture medium (Huang et al. 2012) and genes associated with DNA methylation were negatively correlated with BAP concentration. As reported by Halim et al. (2018), higher histone deacetylase (HDAC) enzyme activity was in general measured in variant plants regenerated from culture media supplemented with high concentration of BAP (4.0 mg/L) alone or combined with IBA. Kinetin also caused extensive DNA hypomethylation in carrot root cultures (Arnholdt-Schmitt 1993).

In relation to gibberellins, in onio, the mitotic index and cell size decreased with increasing concentration of GA_3 (Ud-Deen and Kabir 2009).

2.2 Physical factors

Besides chemical composition of the culture medium, some physical factors may also influence the stability of *in vitro* cultures.

An effect of the temperature on SV has been repeatedly reported (Sivanesan and Jeong 2012; Krishna et al. 2016; Duarte-Aké and De-la-Peña 2021). In *Lilium longiflorum*, development of albino seedlings from somatic embryos increased as the incubation temperature was raised above 10-15ºC (Bhojwani and Razdan 1996). However, in *Senecio cruentus*, SV was observed when the cultures were maintained at 15º, but not at 25ºC (Sivanesan and Jeong 2012). The temperature is known to affect the cell cycle in plants and an inadequate control of the cell cycle *in vitro* has been suggested as one of the causes of SV (Karp 1994).

The physical stated of the culture medium also has been related to occurrence of SV as it influences the cytological behaviour of the cultured cells (Bhojwani and Razdan 1996). In *Hevea*, higher polyploidy level was observed when cells were cultured in suspensions but decreased when they were recultured as callus on a solid medium (Wilson et al. 2006).

Other physical factors such as light intensity, osmolarity, humidity and agitation rate of the culture medium also affect the incidence of SV in plants (Krishna et al. 2016; Duarte-Aké and De-la-Peña 2021). Gas exchange can also play a role in SV due to its influence on ethylene accumulation, which cause epigenetic changes (Karp 1994).

Differences in a number of physical factors can be found in different *in vitro* culture systems and, consequently, in the stress imposed to the cultured cells and tissues. Thus, in Persian lime, analysis by ISSR markers revealed differences in SV frequency depending on the culture system used: semisolid medium, partial immersion in liquid culture medium, and the temporary immersion RITA® system. Polymorphism analysis by using ISSR markers revealed that the highest percentage of variation (26.5%) was obtained in shoots cultivated on semisolid medium. The lowest SV occurrence was observed in shoots propagated in the RITA® bioreactors, where the polymorphism percentage remained below 11% during all the subcultures tested (Bulbarela-Marini et al. 2023). The RITA® system exhibits important differences compared to other culture procedures since it promotes gas exchange and increases contact of tissues with the culture medium (Bulbarela-Marini et al. 2023).

3 *In vitro* culture method

The different *in vitro* culture techniques present important differences at multiple levels. Culture medium requirements significantly vary depending on the propagation method, being especially important the combinations of PGRs, which have a relevant role in SV induction (Armijos-González et al. 2021). The tissue used as starting material and the treatments required for *in vitro* culture establishment are also factors influencing SV that present important differences depending on the *in vitro* culture method. Therefore, the type and magnitude of the stress imposed on cultured cells significantly vary depending on the method used (Krishna et al. 2016) and, in consequence, the nature and frequency of SV.

Different studies have investigated the influence of the *in vitro* culture on SV. For example, in *Oroxylum indicum*, plants directly produced from axillary buds were phenotypically, cytologically and genetically more identical to their mother plants than those indirectly regenerated. While 10% polymorphism by RAPD analysis was found in plants obtained from axillary buds, 24.59% was estimated in plants regenerated via

indirect organogenesis (Gokhale and Bansal 2015). In *Cinchona officinalis*, indirect shoot regeneration induced SV detected by ISSR analysis, whereas direct shoot formation gave rise to true-to-type plants (Armijos-González et al. 2021). Multiplication of *Ophiorrhiza mungos* by direct organogenesis from axillary and terminal buds also gave rise to plants genetically identical to the donor plant, as revealed by the absence of detectable genetic variation evaluated using RAPD markers; however, indirect shoot regeneration induces SV (Kaushik et al. 2015).

In general, axillary shoot proliferation, with direct formation of plant structures from meristems pre-existing in the explant, minimizes the risk of instability (Karp 1994) and it retains clonal fidelity or gives rise to low frequencies of variation (Sivanesan and Jeong 2012; Gokhale and Bansal 2015). In indirect organogenesis, however, SV has been reported in different species, such as *Solanum melongena* (Zayova et al. 2010), *Arracacia xanthorrhiza* (Vitamvas et al. 2019), or *Oroxylum indicum* (Gokhale and Bansal 2015). SV has also been found in somatic embryogenesis systems of different species, such as *Citrus paradisi*, oil palm, rose, potato, grapevine, coffee (Krishna et al. 2016), yacon (Viehmannova et al. 2014), or olive (Leva et al. 2012; Bradaï et al. 2016a, 2019). Nevertheless, many investigations reported genetic uniformity in plants developed using this regeneration method (Fourré et al. 1997; Gallego et al. 1997; Thakur et al. 1999; Harvengt et al. 2001; Jayanthi and Mandal 2001; Pinto et al. 2004; Loureiro et al. 2005; Mallón et al. 2010; Rai et al. 2012). According to Vasil (1987), regeneration via somatic embryogenesis has better chances of obtaining genetically uniform plants than through organogenic differentiation. As proposed by Venkatachalam et al. (2003), it may reduce the frequency of genetic variation in regenerated plants due to developmental constraints during embryogenesis, that select against genetically altered plants.

In general, regeneration methods including a callus phase is likely to provoke more variability than direct pathways, as the callus phase accumulates most of the abnormality generated during *in vitro* culture (Phillips et al. 1994).

Cellular organization is a critical feature determining SV (Rani and Raina 2000). In fact, SV has been related to disorganized growth, and the longer the duration of the disorganized phase the greater the chances of generating SV (Karp 1994). In the same line, the greater the departure from organized growth (Karp 1994) or the more the organizational structure of the plant is broken down, the greater the chance of occurring variations (Araújo et al. 2001; Cooper et al. 2006).

Important differences related to cellular organization are found in the different *in vitro* culture techniques, with methods in which the cellular organization of the initial explant is not altered or minimally changed, such as axillary shoot proliferation or meristem tip culture, to systems in which regeneration is achieved after a phase of disorganized callus or cell suspension culture (Bairu et al. 2011).

Different causes could explain the higher variability found in *in vitro* methods involving a callus phase. Callus formation is considered a culture procedure imposing stress to cells (Smulders and de Klerk 2011), as it involves a dedifferentiation phase, followed by uncontrolled cell divisions, and a redifferentiation process, which can result in genetic and epigenetic changes (Kaeppler et al. 2000; Vázquez 2001; Leva et al. 2012; Grafi and Barak 2015). Moreover, according to Karp (1994), in disorganized growth, the constraints which act to eliminate genetic variations in normal meristems are suppressed, or mechanisms of genetic instability are induced.

The nature of the callus also may affect the variability observed in the regenerated plants (Bhojwani and Razdan 1996). A true callus is a mass of dedifferentiated cells proliferating in an unorganized manner. However, calli composed by a mass of suppressed organs or proliferating embryos, rather than completely unorganized tissue, also develop during *in vitro* tissue culture. While a true callus phase is likely to introduce considerable variability, in these calli it is more likely to retain a high degree of chromosomal stability (Bhojwani and Razdan 1996). This could explain higher genetic stability found in tissue cultures and regenerants of different species, such as asparagus, barley, guinea grass, maize, or wheat (Bhojwani and Razdan 1996).

A variation in the abnormalities detected with the nature of callus has also been reported in somatic embryogenesis systems. In oil palm, whereas slow growing, nodular and compact calli produced 5% variant palms, plants derived from fast growing calli exhibited up to 100% variation (Bhojwani and Dantu 2013). In the same species, significantly different variability was observed between embryogenic and non-embryogenic calli of the same genetic background. According to Giorgetti et al. (2011), it could imply that specific aberrations are strongly selected against in the acquisition of embryogenic competence.

A strong correlation between proliferation rate and SV frequency has been in general established, with higher instability as the multiplication rate increases (Bairu et al. 2006; Martínez-Estrada et al. 2017). In Persian lime, however, this correlation was not observed, as the higher multiplication rate was achieved in the RITA® bioreactors, the culture system producing less SV across subcultures (Bulbarela-Marini et al. 2023). Similarly, in banana, Khan et al. (2011) found an increased number of somaclonal variants as the multiplication rate of propagules decreased.

An interesting point is the evolution of SV during the application of an *in vitro* culture procedure as it may be a helpful tool to predict the moment at which genetic instability is initiated (Lopes et al. 2009). Narváez et al. (2019) investigated SV during somatic embryogenesis in olive by using RAPD markers. Similarly to that reported in *Picea glauca* (DeVerno et al. 1999), they found variations in callus but not in the regenerated plants. According to DeVerno et al. (1999) and González-Benito et al. (2014), embryogenic callus would be formed by a mix of stable and unstable cells, but only cells with an unaltered genome or accumulating not important variations would be able to regenerate plants.

4 Type of explant

Type of tissue used as starting material can affect the frequency and nature of SV (Sahijram et al. 2003; Kawiak and Łojkowska 2004; Chuang et al. 2009; Krishna et al. 2016) Thus, Cao et al. (2016) found differences in the percentage of somaclonal variants in plants regenerated from mature and immature leaves of caladium, with higher rates of SV in plants obtained from immature leaves, independently on the culture medium used. In *Cucumis melo*, a larger frequency of tetraploids was observed in regenerants derived from immature cotyledons compared to those obtained from apical meristems (Adelberg et al. 1994). In rough lemon, plants regenerated from nucellar tissues showed no variation by RAPD analysis, while 20-30% of plants obtained from ovaries showed different banding patterns (Savita et al. 2015).

In general, highly differentiated tissues such as roots, leaves, or stems, produce more variants than explants like axillary buds and shoot tips, which have pre-existing meristems (Duncan, 1996) and no

dedifferentiation is required. Using older or more specialized tissues as explant source also increases the chances of SV in the regenerated plants (Krishna et al. 2016).

As previously indicated, cellular organization plays a main role in the appearance of SV. While in explants with pre-existing meristems, cellular organization is only slightly affected, in other systems, in which dedifferentiation and redifferentiation are required and regeneration is achieved after a callus phase, it is drastically altered (Karp 1994).

Nevertheless, SV has also been found in plants derived from organized meristems (Rani and Raina 2000), vegetative buds (Martínez-Estrada et al. 2017) and nodal cuttings (Soliman et al. 2014). In fact, in bananas, Israeli et al. (1996) reported that shoot-tips caused more variation than somatic embryogenesis. However, this result was attributed to a possible dissociation of chimeras.

Nevertheless, a relationship between the type of explant and the *in vitro* propagation method cannot be ignored as, in general, plant regeneration from differentiated tissues normally takes place directly, from atypical points of origin, or mostly indirectly, through a callus stage (Pijut et al. 2012). In fact, according to Liberatore et al. (2020), the risk of SV when using differentiated tissues as explant source is especially high when indirect organogenesis or embryogenesis occurs, since callus formation entails a dedifferentiation and redifferentiation process, which can result in cellular disorganization and genetic and epigenetic changes.

Different causes can explain differences due to the type of tissue used as starting material. One of them is the existence of pre-existing variations in the tissue used as explant source (Peschke and Phillips 1992). According to D´Amato (1977), gross changes in the genome including endo-polyploidy, polyteny and amplification or diminution of DNA sequences could occur during somatic differentiation in normal plant growth and development. In fact, in some species like oil palm, the genome is naturally unstable, accumulating abnormalities during their growth *in vivo* (Giorgetti et al. 2011). The extent of genetic and epigenetic variations accumulated during normal plant development significantly varied depending of different factors such as the explant age and type of tissue and organs (Bhojwani and Dantu 2013). Thus, SV can arise from somatic variations already present in the mother plant (Karp 1994). This variability present in the tissue used as explant can become evident after *in vitro* culture, probably due to separation of chimeras (Bairu et al. 2011).

5 Length of culture period

The length of the culture period is another factor influencing SV. In general, higher variability was found in cultures maintained for longer periods and their regenerants (Etienne and Bertrand 2003; Bradaï et al. 2016a; Krishna et al. 2016; Bradaï et al. 2019).

Nevertheless, despite the increase in SV normally detected with time in culture, as stated by Tremblay et al. (1999), the effect of culture age is probably underestimated, since a decrease in cultures regeneration ability is usually in parallel observed (Klimaszewska et al. 2009; Park et al. 2011; Bradaï et al. 2016b). Genetic or epigenetic variations accumulated during *in vitro* culture can affect the integrity of the developmental pathway (Brar and Jain 1998), thus compromising its progression. In fact, Wang and Wang (2012), proposed the existence of a certain ‘threshold’ for every plant species, above which, no regenerants would be obtained.

Culture time also affects the nature of the alterations (Plader et al. 1998; Ładyżyński et al. 2002) and the accumulation of abnormalities (Bradaï et al. 2016a). In olive, Bradaï et al. (2016a) found that the percentage of regenerated plants showing morphological variations and the accumulation of variant phenotypes per plant were significantly affected by the culture age, although this effect was strongly influenced by the genotype.

Nevertheless, absence of SV after long-term culture has also been reported. In cassava cultures maintained *in vitro* for 10 years, no polymorphism was found by using Restriction Fragment Length Polymorphism (RFLP) and RAPD markers (Angel et al. 1996). In shoot cultures of *Vanilla planifolia* micropropagated for 10 years, genetic fidelity was also confirmed by using RAPD and ISSR markers (Sreedhar et al. 2007). In these cases, the genetic stability despite long subculture duration have been attributed to their genetic make-up, which could be inherently resistant to alterations due to *in vitro* culture (Chin et al. 2019).

In some investigations, the influence of culture time has been assessed by examining the effect of the number of subcultures. Accordingly with the above, the frequency of variation normally increases with increasing the number of subculture cycles. Thus, during *in vitro* propagation of *Vanilla planifolia*, the lowest polymorphism (0.84%) by using ISSR markers was observed in plants obtained from the first subculture. Shoots from 2-5 subcultures showed less than 4% polymorphism, and percentages of polymorphism greater than 15% were found after the sixth subcultures. The highest variation (19.49%) was detected in plants from the tenth subculture, the latest one tested (Solano et al. 2019). In *Musa*, Rodrigues et al. (1998) showed that somaclonal variants appeared from the fifth sub-culture (1.3%) onwards and increased to 3.8% after 11 subcultures.

In this line, Côte et al. (2001) proposed a mathematical model, by which the occurrence of SV can be calculated as a function of the number of multiplication cycles:

$$\%\, V = [1 - (1 - p)^n]\; x\; 100$$

where, %V = percentage of SV, n = the number of multiplication cycles, p the expected variation probability (the value '$1 - p$' being the expected probability to get a true-to-type plant).

Different causes have been proposed to explain this culture age effect on SV. As previously indicated, *in vitro* culture itself acts as a mutagenic system because cells are subjected to stressful conditions (Jain 2001). Therefore, the longer the culture period, the greater the chances of SV occurrence, with accumulation of new variations as time in culture progress. As proposed by Bradaï et al. (2016a), since almost all the DNA variation arises from DNA replication and cell division *in vitro* (Wang and Wang 2012), higher variation frequencies are expected with prolonged cell proliferation periods. In this line, Brar and Jain (1998) stated that the age effect on increased frequency of mutation was primarily due to sequential accumulation of mutations over time rather than an increased mutation rate in old cultures. Nevertheless, activation of certain types of retrotransposons and increase in retrotransposon copy number (Hirochika et al. 1996), as well as induction of DNA hypermethylation (Rival et al. 2013) have been found in a time-dependent fashion. Accumulation in tissues of some culture media components with a mutagenic effect, like PGRs, can also occurs throughout subcultures, thus contributing to amplify the influence of culture duration (Bradaï et al.

2016a). Additionally, repeated subcultures promote multiplication of the alterations generated in prior cycles (Chin et al. 2019).

There are also evidences pointing to an effect of the frequency of subculture on SV. Investigations in different species, such as alfalfa, carrot, *Nigella sativa*, tobacco, or *Begonia rex*, revealed that frequent transfers yield more stable cultures than long subculture intervals (Bhojwani and Razdan 1996). Increased SV found in cultures maintained through long subculture cycles could be due to different causes as multiple modifications can occur in culture conditions throughout the subculture cycle. Factors like chemical composition (including PGRs), pH, moisture content, or gel strength, can change over time or due to the growing plant tissues. Additionally, different metabolites, such as phenols, can be secreted to the culture medium. These circumstances could increase the stress level at which cultures are normally subjected during *in vitro* culture. Nevertheless, Israeli et al. (1995) reported high SV rates when the period between subcultures is short and more subcultures are performed in a given time.

However, some studies reported lower SV frequencies in plants regenerated from older cultures (Franklin et al. 1989; Wang et al. 1992; Rodríguez López et al. 2010) These results and other counterintuitive findings led to Wang and Wang (2012) to propose a novel hypothesis applying the natural selection theory to *in vitro* cell populations. Based in five premises, they suggested three models for *in vitro* cell selection: a) the selection model for V-win and V-callus, b) the selection model for T-win and T-callus, and c) the selection model for a draw and D-callus.

6 Genotype

The genotype of the parent plant also affects SV. Investigations carried out in different species, such as banana (Reuveni et al. 1993), grasspea (Tripathy et al. 2016), olive (Bradaï et al. 2016b; 2019), black and white spruce (Tremblay et al. 1999), cabbage (Abd-Elhamed 2018), chickpea (Alghamdi et al. 2021), Phalaenopsis (Tokuhara and Mii 1998), oil palm (Eeuwens et al. 2002), or *Coffea arabic*a (Etienne and Bertrand 2003), support this genotype effect on SV.

Important differences in stability have been found among species and within the same species, among cultivars and varieties. Thus, oil palm is an intrinsically variable system, particularly prone to genome instability. According to Giorgetti et al. (2011), natural unsteadiness of the chromosomal and genomic asset is greatly enhanced under *in vitro* culture conditions, giving rise to large genetic instability in the regenerating structures. At an intraspecific level, important differences have been reported among banana cultivars, with overall variation rates ranging from 3% to 20%, depending on the cultivar (Hwuang and Ko 1987). However, in *Phalaenopsis*, the percentage of somaclonal variants ranged from 0 to 100%, depending on the variety (Tokuhara and Mii 1998).

The genotype of the explant donor plant influences both the frequency and type of variation (Bradaï et al. 2016a). Phenotypic analysis of olive plants regenerated via somatic embryogenesis revealed a significant influence of the embryogenic line in the different parameters tested, including frequency of the variant phenotypes, percentage of regenerated plants showing morphological alterations, and accumulation of variant phenotypes per plant (Bradaï et al. 2016a). The results obtained also revealed the existence of genotype-dependent abnormal morphologies, with altered phenotypes only observed in plants derived from

a specific cell line. An important variation in the similarity coefficients determined by SSR and RAPD markers was also found in these plants depending on the genotype (Bradaï et al. 2019).

Although in some cases it is has been reported that the frequency of variation can be genotype-independent (Baklouti et al. 2022), the genotype has been pointed out as a fundamental factor in the appearance of SV in cultures and their regenerants, with genotypes with low, moderate or high risk of variation. In fact, in banana (Reuveni et al. 1993), black and white spruces (Tremblay et al. 1999), and olive (Bradaï et al. 2016a), the genotype has been considered the main factor affecting variability during *in vitro* culture.

Differences in genotype stability are related to differences in the genetic make-up. Some constituents of the genome make it more prone to undergo both genetic and epigenetic changes during *in vitro* culture (Bairu et al. 2011). Genotypes containing transposons are more susceptible to mutate under these conditions than those lacking these elements (Peschke and Phillips 1991). Ploidy level of the explant source can also affect the tendency of cultures to display SV. Generally, ploidy levels lower than the usual ploidy level of the respective species prove to be more or less unstable (Bhojwani and Razdan 1996). Thus, in *Crepis capillaris*, Sacristan (1971) found that diploidization was more common in haploid lines, than the occurrence of tetraploids in diploid lines. Besides, in general, polyploid genotypes tended to show higher variation than diploid genotypes of the same species (Bhojwani and Razdan 1996). Moreover, variations involving changes in chromosome number or structure are more easily recovered in regenerants of polyploids than in those of diploids and haploids, since polyploids can tolerate more gross genomic alterations. However, gene mutations are better expressed in haploids and diploids, although show better survival in polyploids, particularly when such changes are deleterious (Brar and Jain 1998). The genetic make-up can also give rise to a differential sensitivity to the stress caused by *in vitro* culture (Karp 1994), thus resulting in a differential occurrence of SV.

Due to the great influence of the genotype, depending on the objectives of the *in vitro* culture technology, the identification of especially stable or highly susceptible genotypes can be essential. Thus, in *in vitro* methods aiming clonal propagation, it would be advisable to identify highly stable genotypes. However, in order to generate new variability to broaden the genetic basis in breeding programs, highly unstable genotypes could constitute a valuable material for producing sublines displaying interesting traits.

7 Concluding remarks

In vitro culture techniques are increasingly being used due to their multiple applications in different fields, from the industry to basic research. SV is a phenomenon intrinsically linked to cells and tissue culture. However, sufficient attention is normally not paid to SV occurrence and in most cases plant stability is assumed. However, investigations on SV revealed that it is not an irrelevant event but its incidence can be very high depending on the experimental system.

Somaclonal variations occurring during *in vitro* culture are unpredictable in nature and there is no method to control their occurrence Nevertheless, knowing the factors affecting SV can help to “control” its incidence. In this way, *in vitro* culture conditions and other important aspects such as culture duration, the genotype, the type of explant, or the *in vitro* method, can be adapted to promote or reduce SV depending on the objectives of the culture system, focused on maintaining the genetic stability of the plant material or on the generation of new variability for different purposes.

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