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Research Report

In Vitro Breeding of Heavy Metal-Resistant Plants: A Review

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Abstract. Plant biotechnology using in-vitro cell and tissue culture is a practical plant breeding tool in developing plants resistant to different abiotic stresses such as cold stress and elevated soil salinity. In this study, the focus is on the in vitro breeding method applied for development of plants resistant to heavy metal (HM) stress. It consists of the following three successive stages: (i) initiation of callus cells, some of which are somaclonal variants with new traits, (ii) exposure of the calli to HMs as selective agents during proliferation for selection of somaclonal variants with enhanced HM-resistance, and (iii) selection of the desirable resistant variants following plant regeneration in the presence of HMs. The whole procedure is more efficient and cost-effective than the conventional breeding methods. Moreover, the plants developed through this approach are not regarded as genetically modified organisms (GMOs), and therefore, did not pose negative public acceptance issues unlike GM plants. However, despite the numerous advantages of this in-vitro breeding approach, it has been employed in a few plant breeding studies to generate HM-resistant plants. The present study outlined the fundamental principles of in vitro breeding and the progress made so far towards development of HM-resistant plants based on this approach.

***Additional key words*:** abiotic stress, heavy metal tolerance, somaclonal variation, tissue culture

Introduction

Soil contamination with heavy metals (HMs) has become an important public health and food production concern in the past few decades (Sharma and Agrawal, 2005). This problem occurs due to increased human activities such as mining, fertilizer application, and industrial development (McLaughlin et al., 1999). Heavy metals from anthropogenic activities are added to the natural levels released from bedrocks and are persistent in the environment as their potential ecological and health threats cannot be simply removed with the passage of time by microbial degradation. There are many proposed strategies for remediation of soil heavy metal pollution and the use of plants (phytoremediation or phytotechnologies) is of particular interest. Conceivably, the various ways in which plants could be used in the management of heavy metal pollution problems may include: (i) phytoextraction (to reduce soil HM levels), (ii) phytostabilization (to reduce soil erosion, leaching and runoff of HMs by *in situ* immobilization with the help of plant root chemistry and thereby minimizing bioavailability of heavy metals), and (iii) phytovolatilization (uptake by plant roots from soil and then converted by plant cells to volatile forms that can be released into the atmosphere (Pilon-Smits, 2005).

There are many challenges for phytoremediation of soil heavy metal contamination. A basic pre-requisite for phyto-management of soil heavy metal problems is that metal-resistant plants must be used following their identification or development via traditional plant breeding or biotechnology. There is no previous report of in vitro breeding of HM-resistant plants based on somaclonal variation. Therefore, here the fundamental principles and basic methodology of the in vitro breeding approach as applied to development of HM-resistant plants were first outlined. In addition, the few studies showing HM-resistant plants were discussed including characterization of the callus cultures and HM-resistant plants obtained. It is hoped that with a basic understanding of the tissue culture-based development of HM-resistant plants and a discussion of the existing obstacles affecting the efficiency of this in vitro breeding method would stimulate more research into this technology.

In Vitro Plant Breeding for Improved Heavy Metal Resistance

Plants use different strategies to respond to the presence of elevated bioavailable HMs in the environment. The HMs

in soils can be taken up by plant roots and then a portion of the absorbed HMs might be distributed among different plant organs (Mari and Lebrun, 2006). In some plants, even limitation of HM translocation from the roots to the above-ground organs would be of survival value so that photosynthesis could be protected to sustain plant growth (Nocito et al., 2011). Plants may also have evolved HM avoidance or exclusion mechanism so that HM uptake from soils would be reduced or limited (Ahmad et al., 2007; Liu et al., 2009; Seregin et al., 2014; Wei et al., 2005). The HMs are known to induce oxidative stress in plant cells disrupting plant metabolism and growth (Bhaduri and Fulekar, 2012). Therefore, another important strategy is activation of antioxidative enzymes counteracting reactive oxygen species (ROS) produced by HM stress (Hall, 2002; Schützendübel and Polle, 2002) and thereby minimizing the adverse impacts of oxidative stress on the plant cells (Ernst, 2006). Obviously, plants which cannot develop an efficient defense mechanism, can hardly survive in HM-polluted soils. Research approaches that can result in enhanced HM resistance in plants are of particular interest.

Traditional Breeding vs. Tissue Culture-Based Breeding

Although it is well-documented that natural variation in HM uptake and resistance occurs among different plant species and genotypes (Grant et al., 2008), it is not always broad enough to permit selection of the tolerant variants. To improve plant HM-resistance, application of conventional breeding methods can be an alternative but due to the lengthy procedures and high costs required, plant breeders seldom give the HM resistance trait as the priority unlike the more traditional targets such as drought, salinity or biotic stress that affect yield.

In vitro breeding or use of plant tissue culture techniques can induce variation *via* somaclonal variation phenomenon. Therefore, the variant plants obtained are not regarded as GMOs (genetically modified organisms *via* novel gene biotechnology manipulations) as no in vitro-manipulated or recombinant DNA is transferred to generate the variants. Afterwards, variants of interest (with high HM-resistance) can be easily selected through this approach under highly controlled conditions. Unlike traditional plant breeding, there will be a minimal requirement of space and time for in vitro breeding. Indeed, in a recent review on in vitro breeding for abiotic and biotic stresses in plants there are many studies on generation of variants with improved drought, salinity and disease resistance but by comparison only a few HM-resistant plant variants were obtained in this way (Rai et al., 2011).

HMs as Selecting Agents in Plant Tissue Culture Media

In vitro breeding basically starts with explants removed from mother plants grown under in vitro conditions (Fig. 1). Fully-developed plant structures including the leaves and shoots may be used as the initial explants which can be cultured on a common basal plant tissue culture medium such as Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with different combinations and concentrations of plant growth regulators (PGRs). The two principal PGRs often used are auxins and cytokinins which in optimized concentrations can trigger totipotent cells to form undifferentiated parenchyma cells called callus cells (calli) (Gaspar et al., 1996). Callus initiation starts from edges or wounded parts of explant tissues and then can gradually cover whole of the explant surfaces. The appropriate culture conditions such as optimized temperature and lighting are the other requirements provided in a control chamber or growth room. The time and frequency of callus induction can vary with plant species and genotype (Sharma and Agrawal, 2005).

Callus cells are mostly proliferated on an agar-solidified medium containing the same combinations and concentrations of phytohormones as used in the initiation medium. Otherwise, cell suspension culture in liquid medium can be developed but has been used less often in the in vitro breeding literature presumably because establishment and maintenance of cell suspension cultures would require extra efforts and resources (Table 1). At the early stage of callus initiation and proliferation, the chance of somaclonal variation occurrence is very high (Wang and Wang, 2012). It may be advantageous to add a HM of interest in the culture medium at this stage to help to select for any HM-resistant somaclonal variant cells. The callus proliferation stage can take from a few weeks to a few months (Table 1). Prolonged subcultures with stepwise increases in the concentrations of HMs could also help to capture somaclonal variation occurrence. However, the regenerating ability of calli might be adversely affected with prolonged subculture (Bairu et al., 2011; Kaeppler et al., 2000). As the callus cells are not homogeneous, different exposure periods and levels may trigger different responses from the different constituent cell populations in the proliferating callus culture. Some phytohormonal treatments may trigger callus cells to undergo embryogenesis instead of proliferation and the embryos formed may also exhibit HM resistance (Von Arnold et al., 2002).

Calli should be exposed to the stress factors or selecting agents (HMs) during proliferation (subculture) or even from earlier (the callus induction stage) to select for the desirable trait (HM-resistance). Exposure of callus to a sub-lethal concentration of HM has been applied more than stepwise increases in HMs in the previous studies (Table 1). This is

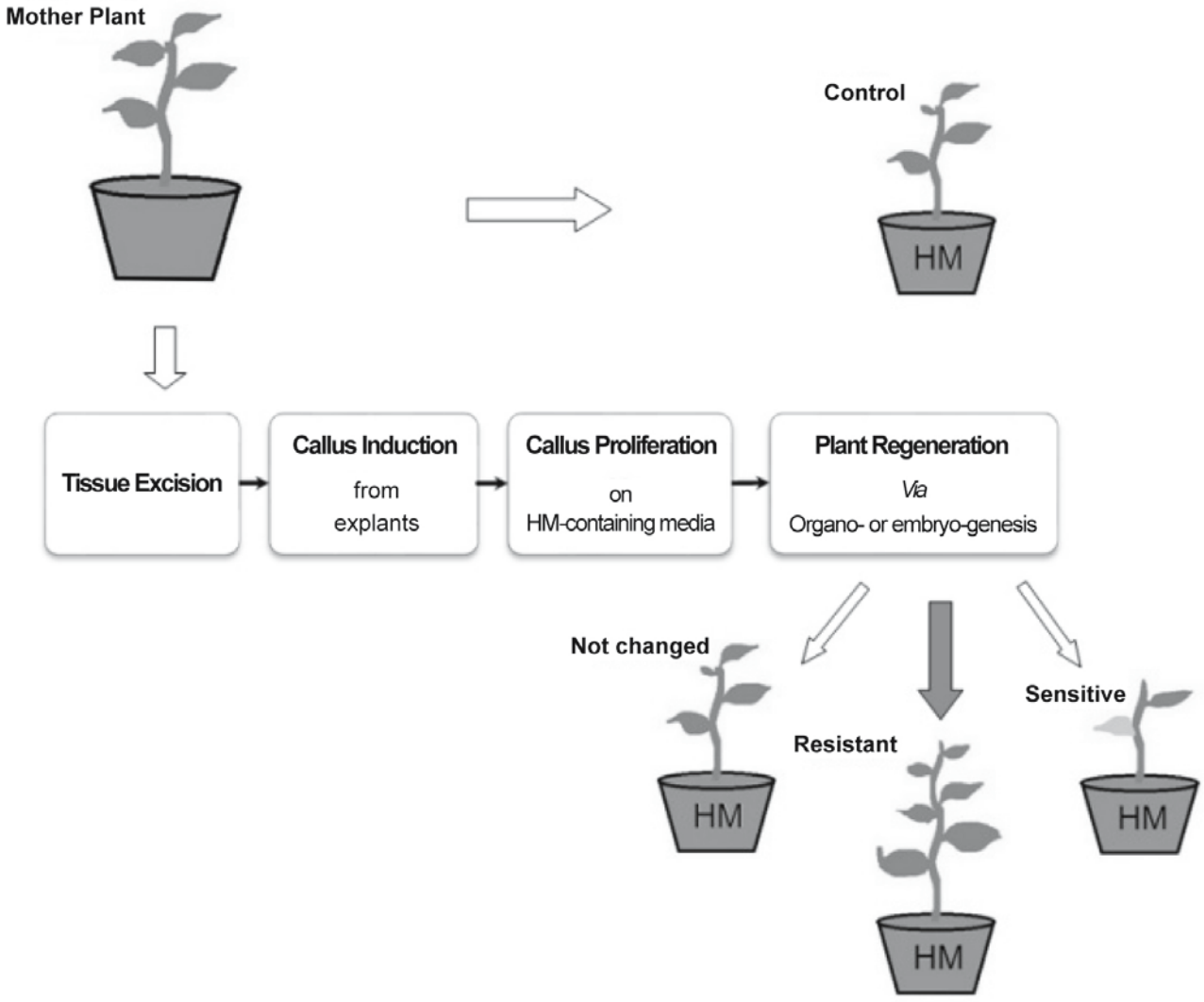

Fig. 1. Schematic procedure of in vitro development of HM-resistant plants. Morphology of progeny may represent the resistance level by comparing with control plants derived directly from mother plants.

probably because during prolonged subculture typical of the stepwise increased HM exposure for selection of HM-resistant plants, a lowering of the regeneration ability of cell culture might result. This would be a significant obstacle for the in vitro approach for plant breeding (Rai et al., 2011). Nevertheless, prolonged selection procedure (4-8 months) was applied in more than one fourth of the previous studies resulting in less than 10 percent shoot regeneration (Table 1) (Van Sint Jan et al., 1997).

Ultimately, to regenerate plantlets from HM-treated calli or embryos, they are transferred to plant regeneration media often lacking HMs (Fig. 1). These media may contain gibberellic acid (GA_3) which is known as a key shoot growth promoter (Gaspar et al., 1996). Although HMs at high concentrations are likely to inhibit plant regeneration, at low concentrations HMs themselves are also known as morphogenesis stimulators by inhibiting ethylene synthesis (Roustan et al., 1989; Rout et al., 1998). Compared to the mother plants cultured under in vitro conditions or plants regenerated from control callus not exposed to HMs, the regenerates from HM-treated calli may show an increased, lowered or same level of HM resistance (Fig. 1).

Identification of Plant Regenerants with Improved HM Resistance

Preliminary identification and screening of the desirable HM-resistant variants are usually possible after in vitro selection and subsequent plant regeneration (Fig. 1) (Bairu et al., 2011; Rai et al., 2011). HM-resistant somaclonal variants may differ from mother plants in terms of morphology, physiology, and biochemical profile. One of the most used methods has been in vitro growth screening of regenerants exposed to substantial levels of HMs as HM stress can affect plant morphology and biomass. In some previous studies,

Table 1. In vitro plant cell selection studies carried out aiming to enhance HM resistance in different plant species. The characteristics of the callus/plants following exposure to the HMs used as selection agents added to culture media were compared to the control. The parameters in a study that showed increase, decrease or not determined are denoted with +, -, and n.d., respectively.

Plant species	Initial Plant material	Heavy metal/ Concentration	Exposure time	Characteristics of HM-resistant callus/plant compared to control				Heritability	Reference
				Dry or fresh weight	Enzyme activity	Metal content	Protein content		
Setaria italic (foxtail millet)	Callus	240 μM Zn	4 weeks	+	+	+	+	+	Samantaray et al., 1999
Echinochloa colona (jungle rice)	Callus	5 mM Cr + 7.6 mM Ni	4 weeks	+	+	+	+	+	Samantaray et al., 2001
Brassica spp.	Callus	240 μM Zn or 800 μM Mn	4 weeks	+	+	+	+	n.d.	Rout et al., 1999
Setaria italic (foxtail millet)	Callus	5.7 mM Ni	4 weeks	+	n.d.	n.d.	n.d.	n.d.	Rout et al., 1998
Cucumis sativus (cucumber)	Cell line	100 μM Cd	6 weeks	+	n.d.	-	-	n.d.	Gzyl and Gwóźdź, 2005
Solanum lycopersicum (tomato)	Cell line	≤6 mM Cd	6 months	+	n.d.	+	+	+	Goldsbrough, 1991
Populus nigra (black poplar)	Callus	150, 250 μM Cd	3 weeks	n.d.	+	+	+	n.d.	Iori et al., 2012
Brassica juncea (Indian mustard)	Callus	10, 20 μM Cd or Pb	6 months	+,-	n.d.	+,-	n.d.	n.d.	Nehnevajova et al., 2007
Nicotiana tabacum (tobacco)	Callus	60 μM Cu	4 weeks	+	+	+	+	n.d.	Rout and Sahoo, 2007
Nicotiana tabacum (tobacco)	Callus	2, 5 mM Mn	8 months	+	n.d.	n.d.	n.d.	n.d.	Santandrea et al., 1998
Oriza sativa (rice)	Callus	250-1000 μM Al	20 weeks	n.d.	n.d.	n.d.	n.d.	+	Van Sint Jan et al., 1997
Oriza sativa (rice)	Callus	87, 175 μM Al	4 weeks	+	n.d.	n.d.	n.d.	+	Roy and Mandal, 2005

some characteristics including root and shoot lengths, number of leaves, fresh and dry weights, HM concentrations, protein contents, and activities of antioxidative enzymes were analyzed (Table 1). It seems that many HM-resistant variants exhibited higher rates of growth and development in the presence HMs compared to the plants from which explants were obtained for the callus cultures used to generate the HM-resistant plants (Table 1). In agreement with other studies in the literature, HM-selected somaclonal variants exhibited increased activities of antioxidative enzymes such as catalase and peroxidases upon exposure to HMs. This supports the notion that antioxidative defense is a main plant strategy to minimize the adverse impacts of HM-induced oxidative stress.

Screening of in vitro-selected HM somaclonal variant plants may also be carried out under hydroponic conditions. For example, Nehnevajova et al. (2007) observed three different phenotypes of *Brassica juncea* with improved HM-resistance cultured hydroponically in a growth chamber. The responses of some HM-selected somaclonal Indian mustard plants to Cd and Pb were different, suggesting that the differential resistance of the HM-somaclonal plants to Cd and Pb.

Characteristics of HM-Resistant Somaclones

The HM-resistant plants obtained under in vitro conditions may have different resistance mechanisms or attributes compared to the mother plants from which explants were used to initiate callus cultures for isolation of somaclonal HM-variants (Clemens, 2001; Zhu et al., 2007). For instance, increased HM accumulating ability in above-ground plant parts is desirable for breeding to improve hyperaccumulator species intended for cleaning up HM-contaminated soils (phytoremediation). However, this can be a trait of concern as far as food crop species are concerned as this may threaten food safety (Van der Ent et al., 2013). Some of the somaclonal variants of food crops obtained under in vitro conditions could possess this trait. Therefore, trace element, biochemical, and molecular analyses can be helpful to characterize and distinguish the HM somaclonal variants of interest in terms of uncovering the possible HM resistance mechanisms that may operate in them (Table 1). Many genetic and epigenetic mechanisms may be responsible for somaclonal variation (Lestari and Endang Gati, 2006; Rai et al., 2011). DNA sequence alternations such as point mutations can generally be passed onto the progeny (Bairu et al., 2011). Performance evaluation and heritability studies on the selected HM-resistant somaclones under field conditions will be necessary (Rai et al., 2011). It has been highly recommended in the literature that the stability of the resistance trait should be ascertained to make these new plant lines usable for agricultural applications (Nehnevajova et al., 2007; Roy and Mandal, 2005; Santandrea et al., 1998; Van Sint Jan et al., 1997). However, no long-term heritability screening has been applied in most previous works on selected HM-resistant somaclones and most studies were limited to only one generation. As it is outlined in the Table 1, the HM resistance character in some HM-resistant plants obtained through in vitro breeding was found to be inheritable. The

HM-resistant plants from this approach should be more acceptable to the general public than transgenic plants (genetically modified organisms) since all the HM-resistant somaclonal plants like those variants from natural evolution can be obtained without the deliberate introduction of foreign DNA (Predieri, 2001).

Metal uptake ability can also vary among the selected variants. However, as it is depicted in Table 1, the resistant variants are more likely to show increased HM uptake potential. There was a correlation between HMs and protein contents in plants which may be related to the key role of HM binding-proteins or peptides such as phytochelatin (PC) in the process of HM sequestration into vacuoles for minimizing the impacts of HM-induced oxidative stress on the cytosol. Approximately 90 percent of Cd ions in high Cd-resistant tomato cells were found bound to PC (Goldsbrough, 1991). On the other hand, tolerant cucumber cell lines selected on medium containing 100 μM Cd showed reduced Cd uptake ability when exposed to the same level of Cd for up to 5 days (Gzyl and Gwóźdź, 2005). Likewise, more than 50 and 70 percent of the selected Indian mustard variants were identified as Cd and Pb excluders, respectively (Nehnevajova et al., 2007). The selected variants seemed to be more resistant to exogenous Pb than to Cd.

Proper characterization of newly developed plants is one of the main requirements before they can be released to the agricultural industry for use. To the best of our knowledge, none of the available HM somaclonal variants has been commercialized yet which can be mainly due to this problem. For example, in the first four studies summarized in Table 1, the resistance of the regenerants to HM exposure following their regeneration was not evaluated (Rout et al., 1999, 1998; Samantaray et al., 1999, 2001). Finally, assuring the public that the new HM-resistant somaclonal plants are not GMOs is an important step towards practical application of these new plants, particularly in the case of crop species. To achieve this, molecular genetic characterization of the new HM-resistant somaclonal plants particularly in relation to the possible molecular genetic basis of the variant HM resistance character and the possible molecular mechanism(s) leading to the resulted somaclonal variations will be needed.

Conclusion

As it is outlined in the present study, application of plant cell and tissue culture has not been limited to only micropropagation in higher plant but is also the basis of in vitro breeding applied to obtain plants resistant to abiotic stresses such as HM stress. In vitro breeding is considered as one of the most publically acceptable breeding methods since it is able to broaden genetic variation (*via* somaclonal variation) without any gene transformation. Hence, the new HM-resistant plants developed through this method should not attract the public debate which has plagued the introduction of GMOs (Predieri, 2001). Besides, there will be numerous interests in non-GM plants resistant to HMs with acceptable yield particularly in some countries including New Zealand, Japan, South Korea, and Germany (Veyssiere, 2007). Moreover, it is possible to select the non-GM plants resistant HMs that are also excluders or low accumulators of HM, particularly in the edible parts such as seeds or tubers, etc. In vitro selection of variants resistant to abiotic stress is possible under controlled laboratory conditions making the efficiency of this method higher than conventional breeding methods in terms of time, space, and cost. However, despite all of these remarkable advantages, it has been barely used to develop HM-resistant plants.

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