EURASIAN JOURNAL OF AGRICULTURAL RESEARCH

Tissue Culture Applications in Lettuce (*Lactuca sativa* L.)

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Abstract

Lettuce (*Lactuca sativa L.*) is a major fresh leafy vegetable and belongs to the *Asteraceae* family (Compositae). The focus of modern lettuce breeding objectives is to improve horticultural characteristics such as quality, resistance to early bolting and breeding for resistance to pests and diseases. These characteristics may be improved using biotechnology and gene transfer strategies. Tissue culture methods have proved successful in rescuing selected lettuce genotypes and producing seeds in a disease-free environment. And also overcome the limitations of *in vivo* techniques by shortening period of breeding programmes. The genetic engineering of lettuce requires a reliable and efficient tissue culture methods. Among these methods haploidization is of great interest for producing pure lines. Shoot organogenesis may aid the use of genetic engineering to improve important characteristics of lettuce. Embryo rescue is an important application for transfering the resistance to downy mildew, *LMV*, and beet western yellows virus from *L. virosa* and *L. saligna* into cultivated lettuce. Somatic hybridization via protoplast fusion has also been used to gain access to exotic, sexually incompatible germplasm. Propagation from axillary and apical buds of lettuce plants can also be routinely carried out. If propagation through tissue culture can be done efficiently on a large scale, it may be used to produce F_1 hybrid plants and fix the hybrid vigour. Somaclonal variation provides another source of genetic variability. Regeneration via Somatic embryogenesis has advantages for lettuces.

Keywords: Lettuce, Somatic embryogenesis, Haploidization, Shoot organogenesis, Somaclonal variation, Somatic hybridization

INTRODUCTION

Lettuce (*Lactuca sativa* L.), a fresh leaf vegetable, is a prominent vegetable for consuming and harvesting. They are a self-fertilising species that can be produced yearly, with cultivation being possible all over the world. While the beginnings of lettuce can be traced to the Middle East and south-west Asia, today they are mainly produced and consumed in Europe and the United States (Ryder, 1986). Different regions can have different preferences in colours, shapes, sizes, and flavours. In 2016, Turkey produced 233 662 tons of butter-head lettuces, 179 712 tons of normal lettuces, and 65 068 tons of icebergs (head salad) (Anonymus, 2017).

Due to the high nutrition level found in lettuce and the health benefits resulting from them, especially when eaten in freshly made salads, their production rate continues to increase daily. However, different varieties that are adapted to various growing conditions and seasons may be needed (Siddiqui, 2014). Thus, for the successful cultivation of lettuce (*Lactuca sativa* L.), from the germplasm of their field-grown versions, they must be assessed, selected, and rescued in a short amount of time. For this purpose using tissue culturing methods have been shown to result in efficient rescuing of the lettuce genotypes that were chosen and

consequently developing seeds in environments free from disease (Jenni et al. 2006). Because of the significance of lettuce, breeders have utilised various tissue cultures (Pink et al. 1987; Ampomah-Dwamena et al. 1997) and genetic manipulation techniques (Kim et al. 2004) with every technique needing a certain amount of totipotency for a favourable outcome. To develop better crops by manipulating novel characteristics, both *in vivo* and *in vitro* techniques are involved. For decreasing the long times of breeding programmes, which is a limitation of *in vivo* techniques, *in vitro* techniques like anther and cell suspension cultures from callus, tissue culture, shoot organogenesis, somatic hybridization by protoplast fusion, and somatic embryogenesis can be applied. This is because the *in vivo* techniques use up a lot of time and has the problem of pre and post-zygotic sexual incompatibility between species (Matsumoto, 1987). In this paper, we will summarize the tissue culture methods used in lettuce.

Somatic Hybridization Via Protoplast Fusion for Lettuce

In 1982, field testing and selection for desirable characteristics in head lettuce plants derived from protoplasts was done for the first time Engler and Grogan, 1982). For an efficient method to remove the difficulties associated with interspecific crossing, somatic hybridization by protoplast fusion can be used. This technique has recently been successfully implemented for raising new traits in new varieties that were a demand in the market for the *Lactuca* species (Siddiqui, 2014). Polyethylene glycol and electric methods can both be used to obtain lettuce protoplast fusion easily (Taniguchi, 1990). Somatic hybrids between *L. sativa* and *L. serriola* were acquired (Matsumoto, 1987). From lettuce, *Lactuca sativa* L., developed in greenhouses and growth chambers, and from shoots, roots, and cotyledons of seedlings developed *in vitro*, protoplasts can be obtained (Berry,1982). Between Evola and Red Leaf Amboni lettuce cultivars, green and red respectively, somatic hybridization was conducted so that the investigation on the culture could be carried out in four media types by polyethylene glycol (PEG) and electro fusion. While other treatments were observed to damage the protoplast, it was shown that chemical and electro- fusion of both micro and macro colonies (where in petri dishes there were 5 to 73 average micro colonies) resulted in significant outcomes for fused protoplasts when treated with the T4 treatment for each replication. Additionally, electro fusion was shown to result in protoplast fusion of 40.51% frequency over PEG (Siddiqui, 2014).

By utilizing universal hybridizers containing both dominant and recessive characters, it has become much easier for selecting somatic hybrids from some model plants. The development of backcross progenies with *L. sativa* is ongoing for female fertile plants. Due to various desirable traits like virus, fungi (*Bremia lactucae*), and bacteria resistance have been identified in wild *Lactuca* species, it now seems that using somatic hybridization in breeding programmes is an efficient possibility (Chupeau et al. 1994).

For obtaining germplasm that are exotic and sexually incompatible, protoplast fusion has been employed. It was recorded by (Mazier, 1999). That hybrids between *Lactuca sativa* and two wild *Lactuca* species, *L. tatarica* and *L. perennis*, were acquired via the protoplast technique. However, there was a significant limit in the development of useful plant material due to the regrown plants having very low fertility even when numerous backcross generations were produced. In addition, hybridization of *L. sativa* and *L. virosa* somatically was produced but with all the developed plants showing sterility (Matsumoto, 1991). Although the development of offspring of the other wild species that were investigated, like *L. perennis*, *L. viminea*, *L. juncea* and *L. tatarica*, was not found to be successful, even with many attempts of pollination via different breeders using numerous lettuce lines and different

wild specie accessions. *In vitro* rescue of immature embryos obtained from interspesific hybridization between *L. sativa* and *L. virosa* was reported to be successful when performed under tissue culture conditions. For obtaining vigorous hybrid plants, hybridizaton of *L. sativa* and seven accessions of *L. virosa* were performed. The regeneration of somatic hybrids between *L. sativa* and either *L. tatarica* or *L. perennis* was performed through protoplast fusion and as final outcome of *L. sativa* and *L. tatarica* hybrids were backcrossed to *L. sativa* (Maisonneuve et al. 1995).

Somatic Embryogenesis for Lettuce

One of the useful ways for large-scale propagation of plant material is propagating by somatic embryogenesis *in vitro*. However, for the production of lettuce, there is still no advance in a full protocol development for somatic embryogenesis. Two explant sources (sectioned and whole cotyledons) were developed so that somatic embryogenesis could be initiated. All induction, proliferation, maturation, and conversion of the somatic embryos were satisfactory, particularly in liquid systems, for the mass production of the Paris White lettuce genotype with regards to potential commercial applications (Pinheiro, 2012). Both juvenile and mature tissues could be used as well as explants taken from a wide range of plant species to develop somatic embryos (Seabrook and Douglass, 2000).

A two-step protocol was reported (Seabrook and Douglass, 2000) for the redevelopment of somatic embryos for a wide range of potato (*Solanum tuberosum* L.) genotypes, ploidy and tissues, *in vitro*. Seven cultivars of lettuce were used to redevelop somatic embryos, using these protocols.

For the redevelopment of the somatic embryos, excisions of stem, petiole, and leaf tissues were utilized. Even though stem-internode sections were mainly more productive, the lettuce explant (stem-internode, leaf, and petiole) seedlings from all the cultivars showed redevelopment. While, leaf and petiole explants was the most productive for redevelopment from mature lettuce tissues (Seabrook and Douglass, 2000).

An effective method for using three commercial lettuce cultivars (*Lactuca sativa* L. cv. Great Lakes 659-700, Salad Bowl, and Prize Head) from which cell suspensions were obtained directly and shoots were regenerated, was reported by Teng et al. Among the factors investigated for effecting cell growth and differentiation of the suspension culture (which consisted of callus quality, light intensity, carbohydrate type and concentration, auxins, and cytokinins), it was found that callus quality and carbohydrates were the most important. SH (Schenk and Hilderbrandt) basal medium with 1000 mg myo-inositol/L, 1.5% glucose, 0.44 μM BA, and 0.54 μM NAA was found to be the best medium for the regeneration of shoots in the suspension culture with a 5.8 pH. With these conditions, it was observed that from cell aggregates of 50 to 55 mg (dry weight), hundreds of shoots were developed within two weeks (Teng et al. 1992).

Haploidization for Lettuce

More productive breeding tools are needed for programmes for the improvement of plants. For the development of pure lines haploidization is of great importance amidst these (Piosik et al. 2016).

For the development of embryos to be induced with *L. sativa* maternal genome, the distant pollination technique was applied from the various haploidization methods that have been known to be successful in other species. Pollen grains from 25 different species were utilized for encouraging the process of parthenogenesis in lettuce to begin. The crosses between the *H. annuus* and *H. tuberosum* species pollen were found to be the most productive among these, and they were also used for the ensuing experiments (Piosik, 2013).

For deducing an effective method for haploidization in lettuce (*Lactuca sativa*), chemical treatment of pistils or wide crossing of lettuce with 25 species (*Asteraceae* family mostly) were carried out to start the development of haploid embryos. Crossing with *Helianthus annuus* (16%) or *H. tuberosum* (19%) resulted in the highest embryo frequencies. With all the embryos that showed globular or heart stage development, it was observed that they were haploid (n=9) (Łukasz, 2014; Mazier et al. 2003).

As there was no further development observed in the haploid pro-embryos after pollination, even with cellular endosperms being present, distant pollination using *Helianthus annuus* L. or *H. tuberosus* L. fresh pollen grains were carried out *in vivo*. This resulted in the regeneration of 23 haploid *L. sativa* plants (Piosik et al. 2016).

In Vitro Evaluation Of Disease Resistance In In Vitro Cultivated Lettuce

By examining *Lettuce Mosaic Virus* (*LMV*) infections on either newly regenerated plantlets or *in vitro* cultivated seedlings *in vitro*, the possibility of developing a new inoculation and propagation method for *LMV* infection of lettuce (*Lactuca sativa* L.) was examined (Mazier et al. 1999). They found that inoculating lettuce plants *in vitro*, under sterile conditions, with *LMV* was both possible and not difficult to do (Mazier et al. 2003). It was also shown in another study that, tolerance to downy mildew, *LMV*, and beet western yellows virus could be transferred to cultivated lettuce (*Lactuca sativa* L.) from *L. saligna* and *L. virosa* (Maisonneuve, 2003) Koyama et al. developed a universal method for lettuce tipburn susceptibility testing, *in vitro*. For verifying the dependability of the *in vitro* results for hydroponic experiments, two cultivars were chosen (Koyama, 2012).

Interactions of Genotype And Culture Medium Affecting Shoot/Root Organogenesis

Both plant growth regulators and genotype together highly effects the regeneration of shoots in lettuce (Doerschug and Miller, 1967). were the first researchers to report the successful regeneration of shoots from cotyledon explants which were cultivated in a medium with 5 mgl^{-1} IAA and 0.5 mgl^{-1} kinetin. In another study, the effects of different concentrations of NAA and BAP on the induction of callus, average number of shoot regeneration per leafy explant, and shoot-producing explant percentage were studied. It was observed that under low NAA and BAP concentrations, lettuce explants resulted in low callus formation and direct shoot regeneration increased (Latif et al. 2014).

Lettuce plants developed in fields, which were chosen for seed production, were used for breeding healthy plants by constructing a tissue culture method in which Murashige and Skoog's (MS) medium was used. For the successful development of shoot growth from axillary bud explants (2-3 mm long), a medium consisting of MS + 1.0 or 2.0 mgl^{-1} kinetin and 6.4 mgl^{-1} IAA was used. Although, kinetin of 0.5 and 4.0 mgl^{-1} concentrations resulted in poor shoot growth. After transferring the cultures form MS + 1.0 mgl^{-1} kinetin to MS + 6.4 mgl^{-1} IAA and from MS + 2.0 mgl^{-1} kinetin to MS + 4.8 mgl^{-1} IAA, successful rooting was

observed after 3 to 4 weeks. Additionally, 3.2 and 8.0 mgl^{-1} IAA concentrations resulted in poor initiation of roots. It was also observed that for the cultures developed at 40 Wm^{-2} of light there were more root initiation than those in 5 Wm^{-2} of light. Once the rooted cultures were planted and formed in compost with a success rate of 90-95%, the regenerated plants flowered after 18 weeks [5]. Due to explants that are incubated under light resulting in the development of more shoots, light is an important factor for shoot regeneration (Webb et al 1984).

For the identification of genotypes, especially those that are susceptible to genetic manipulation at the cellular and molecular level, screening of lettuce cultivars comprising of a large variety that represent the four main morphological types has occurred. [30] and [6] researched how a media with 0.1 mgl^{-1} IAA, 0.5 mgl^{-1} kinetin and 0.05 mgl^{-1} zeatin effected the genotypes of lettuce for the regeneration of shoots from cotyledon explants. Regenerating plant genotypes in tissue cultures is the key to success for these methods involving genetic manipulations. It was also stated by Vanjildorj et al. that using cotyledon explants of lettuce to result in shoot regeneration successfully in media containing 0.5 mgl^{-1} kinetin and 0.05 mgl^{-1} NAA was also possible (Vanjildorj, 2005).

Additionally, it was reported that by using media with 0.54 μM NAA and 0.44 μM BA for culturing isolated cotyledon explants, the percentage of explants developing shoots and the mean shoot number per explant doubled after 3-14 days once germination occurred as opposed to other combinations of auxins ad cytokinins (1= 28.54 μM IAA, 32 μM kinetin, 0.23 μM zeatin; 2= 0.57 μM IAA, 2.32 μM kinetin; 3= 0.57 μM IAA, 0.44 μM BA) (Hunter and Burritt, 2002). While in another study, after a number of different types of media were used for studying five different genotypes, a high number of regenerations (more than 2 shoots per cotyledon explant) of shoots were observed when a medium consisting of 0.1 mgl^{-1} NAA and 0.1 mgl^{-1} BA was used (Kanamoto et al. 2006).

For the initiation of callus formation (Dias et al. 2006), after seeds were germinated for 48 hours, cultured cotyledons and placed the callus into a MS medium which had 0.1 mgl^{-1} BA and 0.1 mgl-1 IBA added to it, after which the callus, for the indirect regeneration of shoots, was then placed into MS media with 0.1 mgl^{-1}. After various combinations of NAA and BAP were studied for observing their effects on the induction of callus and the regeneration of shoots from lettuce cotyledon explants, it was detected that at low concentrations of BA, the most number of direct regeneration of shoots occurred (Mohebodini, 2011).

CONCLUSIONS

Industrial and agricultural applications have taken precedence in research conducted on tissue cultures in the past decade. Among the applications that have been successful in tissue culturing, the most important have been somaclonal and gametoclonal selection of variants to produce new varieties, utilizing androgenises routinely for programmes related to plant breeding, using genetically manipulated cells for transgenic plant regeneration, industrial compound development, and crop plant and endangered species germplasm preservation. Various factors have an effect on the responses observed by tissue cultures among experiments, with some not being measurable nor controlled (George, 1993). A diverse response amidst genotypes was indicated by the regeneration of shoots in culture. It was recently determined that for the regeneration of a large order of lettuce genotypes, an enhanced culture medium was possible (Xinrun and Connor, 1992).

Lettuce haploids supply a good foundation for the development of double-haploid (DH) lines, and the production of novel varieties of the common lettuce species can be possible in the future (Piosik et al. 2016). Additionally, from the area near cotyledon petioles, direct regeneration of shoots could help by improving characteristics of lettuce that are valuable via aiding in genetic manipulations (Mohebodini et al. 2011). For lettuce regeneration, it was observed that somatic embryogenesis was effective. For starting material employment a better stock plant should be used for clonal propagation from plant mature tissues (Seabrook and Douglass, 2000). Interspecific hybrids were developed successfully between *L. sativa* and wild *Lactuca* species by using an immature embryo *in vitro* culture and somatic hybridization (Siddiqui, 2014; Jenni, 2006, Chupeau, 1994; Maisonneuve, 1995). It can be expected that utilizing the aforementioned techniques for breeding purposes, could, in the future, assist in increasing lettuce variability.

ACKNOWLEDGMENT

This manuscript was presented as abstract at the International Conference on Agriculture, Forest, Food Sciences and Technologies conference held in Cappadocia / Nevşehir on May 15-17, 2017.

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