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DEVELOPMENT OF *IN VITRO* CULTURE TECHNIQUES FOR ADVANCEMENT OF RYE (*SECALE CEREALE* L.) BREEDING

JANUSZ ZIMNY AND KRZYSZTOF MICHALSKI*

Plant Breeding and Acclimatization Institute - National Research Institute, Poland

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Rye is an important crop widely cultivated in Europe, but one of the hardest to improve due to its allogamy and self-incompatibility. The market for rye-based products is constantly growing thanks to the popularity of organic farming, feed production and diverse industry applications. To address these demands, new highly productive hybrid rye varieties are needed. Currently, full potential of heterosis in rye breeding is hard to reach due to the limited success in *in vitro* cultures. This review summarizes the progress in rye *in vitro* cultures and proposes novel approaches to overcome recalcitrance in this species.

Keywords: breeding efficiency, homozygous lines, haploids, hybrid varieties, *in vitro* cultures, review, rye, *Secale cereale*

INTRODUCTION

The genus *Secale* includes 14 species, but only *Secale cereale* L. plays an important role in agriculture (Vasquez and Linacero, 1995). It is an allogamic and self-incompatible species. Only two representatives of the rye genus: *S. vavilovi* and *S. silvestre* are self-pollinating (Rybczyński, 1990). *S. cereale* was a donor of the R genome in triticale (×*Triticosecale* Wittm.) and is widely used to increase diversity in that synthetic species. Many rye-wheat chromosomal translocations were produced where triticale was used as a bridge species for introduction of the valuable rye genes into the wheat breeding programs (Lukaszewski, 2015). Rye is traditionally cultivated in Central and Eastern Europe and in Scandinavia. Winter hardiness and good adaptability to light and acidic soils, predominant in these regions, are key features of the species. Rye bread is one of the re-discovered regional products and a strong point of organic farming in Europe. Its production requires distinct techniques for making dough due to the lack of gluten – a mixture of proteins specific for wheat. The baking value of wheat largely depends on water-binding properties of gluten (Dwars and Siebel, 2001), whereas at least one out of rye storage proteins, secalin Sec-1, significantly decreases the quality of bread. Besides being used in bread-making, rye is gaining popularity in brewing and distilling industries. Several papers considering rye malting properties have recently been published (Hubner et al., 2010; Jin et al., 2018; Wang et al., 2018). Rye is mostly used for human consumption as well as for feed and biomass production, with dual purpose systems being in development (Ates et al., 2017).

Due to the allogamy and self-incompatibility of rye, until the last decade, most of the cultivars registered in the European Union were open-pollinated varieties. However, the number of hybrid varieties is now growing. Hybrid rye breeding involves crossing a male sterile homozygous parent line with a line designed to restore fertility. Such crossing leads to hybrid vigor in the F1 generation. Out of 65 rye varieties being currently registered in Poland, 24 are hybrids, but only 3 are of Polish origin. By the end of 2018, among 195 rye varieties collectively registered in the EU, 28 were hybrids (according to European Commission's Plant Varieties Database). Leading European breeding companies seem to be particularly interested in the development of hybrid varieties. For exapmle, KWS Saat, a breeding company, offers 13 hybrid rye varieties in the EU, which corresponds to nearly 50% of the company's rye portfolio.

In pursuit of improved breeding material, scientific institutions and breeding companies are strongly interested in quick plant propagation,

* Corresponding author, email: krzysztof.michalski@ihar.edu.pl

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homozygous lines production and genetic engineering techniques. Previously, homozygous lines were produced by multigenerational inbreeding, which in rye is particularly difficult because of its self-incompatibility and inbreeding depression (Hoffmann and Wenzel, 1981). Pure lines that do not segregate in subsequent generations are not only an integral part of modern breeding programs, but can also be used in basic research. An effective method of doubled haploids (DHs) production could accelerate hybrid rye breeding. Unfortunately, among cereal crops, rye is considered to be one of the most recalcitrant in *in vitro* regeneration (Haliloglu and Aydin, 2016). This applies to both somatic tissue regeneration and the gamethophytic cells reprogramming to sporophyte development.

Considering the above facts, one of the major objectives of research should be development of efficient protocols for the *in vitro* regeneration of rye.

IN VITRO CULTURE OF RYE SOMATIC TISSUE

In vitro cultures of cereals are generally considered to be difficult and initially regeneration of such species was thought to be impossible. In the 1950s and later in the 1970s attempts of rye regeneration from root cultures were reported, resulting in callus growth (Roberts and Street, 1955; Carew and Schwarting, 1958; Mullin, 1970). So far, nobody has managed to regenerate full plants using rye roots, although it is possible, e.g., in rice and barley (Zimny and Lörz, 1986b; Chand and Sahrawat, 2000). Since the importance of meristematically active tissues in regeneration was proven (Dale, 1983), several explants at different stages of ontogenesis were used in rye *in vitro* cultures. A morphogenetic potency of embryo tissues of di- and tetraploid rye was pointed out with the scutellum to be best suited for callus induction as well as for plant regeneration (Rybczyński, 1978a, b; 1979). Plant regeneration from mature and most importantly from immature embryos has been reported (Lu et al., 1984; Rybczyński and Zimny, 1985; Ward and Jordan, 2001). Other papers describe somatic embryogenesis from the leaf base (Linacero and Vasquez, 1986; Zimny and Lörz, 1986a; Holiloglu and Aydin, 2016) and immature inflorescence cultures (Krumbiegel-Schroeren et al., 1984; Eapen and Rao, 1985; Zimny and Lörz 1986a; Rakoczy-Trojanowska and Malepszy, 1993). Somatic embryo formation is usually induced by auxin added to culture media, with 2,4-dichlorophenoxyacetic acid (2,4-D) and dicamba being most commonly used (Zimny and Lörz 1989; Eudes et al., 2003). Callus induction from rye inflorescences, the establishment of a cell suspension culture and plant regeneration from suspension cell-derived protoplasts were reported by Ma et al. (2003). However, only 7% of the transferred embryogenic protocalli produced green plants. Endosperm-supported mature embryo culture was described by Birsin and Özgen (2008), giving higher regeneration efficiency than immature embryos in all tested genotypes.

Somatic tissue culture and regeneration enable quick multiplication, cryopreservation of important genotypes, genetic transformation and many other applications. Negative phenomena related to *in vitro* culture of somatic rye tissue might be observed. Part of the regenerants was reported to be sterile (Lu et al., 1984; Eapen and Rao 1985). Somaclonal variation (Linacero and Vazques, 1993; Puente et al., 2008) and albinism (Linacero et al., 2011) are also common among regenerants, which is presumably associated with genomic DNA methylation (González et al., 2013). Aydin et al. (2016) tested the correlation between somaclonal variation and the type, as well as the concentration of auxin used in the culture. The authors demonstrated that relatively more epigenetic than genetic changes can be observed in the rye culture. An increasing concentration of 2,4-D and picloram led to hypermethylation, whereas dicamba caused hypomethylation. Genetic changes were also more frequent when a higher concentration of auxin was used. Nevertheless, dicamba was observed to cause substantially fewer alterations, suggesting this type of auxin to be more suitable for rye cultures.

Somatic embryos were postulated to be formed from a single cell (Esau, 1977). The idea that a transformed cell could be regenerated into a uniformly transgenic plant was confirmed experimentally in the 1990s, when the first transformants of many cereal species were produced. Among others, transgenic rye was obtained using particle gun bombardment (Castillo et al., 1994; Sowa et al., 2000). Popelka et al. (2003) described a method for immature embryo bombardment with subsequent selection of low transgene copy rye plants. At the same time, the system of rye genetic transformation based on immature embryos co-cultivation with *Agrobacterium tumefaciens* and subsequent shoot primordia induction were described by Popelka and Altpeter (2003). The cited reports show, that rye might be directly improved by genetic transformation. Nevertheless, the use of transgenic plants in the EU is strictly regulated and the maize MON810 is the only variety that is allowed for cultivation. On the other hand, transformation will undoubtedly play a major role in the basic research and in the development of new breeding techniques.

PRODUCTION OF RYE HAPLOIDS

The DHs production is more problematic in an out-crossing species than in self-pollinating ones. The phenomenon of close pollination historically has been linked to the androgenesis performance. It appeared that the majority of successful work on androgenesis in rye was performed using lines descending from partially self-pollinating *S. vavilovi* (Wenzel et al., 1977; Friedt et al., 1983; Flehinghaus-Roux et al., 1995). Due to the low survival rate of green regenerants and low fertility in rye, only a fraction of the produced DH plants can be used in research and breeding. Due to the recombination, each microspore carries a different combination of the parental genetic material. Thus it is possible to regenerate plants with a broad range of unique combinations of genes, resulting in the material showing a high level of variability. With a wide pool of variable regenerants, the most desirable genotypes can be selected for traits such as efficient fertility restoration, high yield, stress resistance and others. Rye anthers compared to other cereals are relatively long and contain many microspores (ca. 22 000; Piotrowska, 2008). Microspores can be induced to switch their developmental pathway, resulting in the production of haploid embryos and then plants (Immonen and Anttila, 1996). However, the use of highly morphogenic plant material is crucial for the regeneration efficiency (Tenhola--Roininen et al., 2006).

Attempts of rye anther culture usually resulted only in the initiation of cell division and possibly obtaining callus (Malepszy, 1975; Orlikowska, 1977) or non-germinating embryos (Zenkteler and Misiura, 1974). Despite failures reported by many researchers, successful rye plant regeneration was achieved (Thomas et al., 1975; Wenzel et al., 1975). Subsequent studies showed the impact of various factors on the embryo induction and green plants production from microspores (Flehinghaus et al., 1991; Flehinghaus-Roux et. al., 1995; Rakoczy-Trojanowska et al., 1997; Immonen and Anttila, 1999; Mikołajczyk and Broda, 2000). Many years of research led to the establishment of methodology for obtaining rye doubled haploids (Immonen and Tenhola-Roininen, 2003), but only for the specific genotypes.

Papers published between 1996 and 2012, starting with Immonen and Anttila (1996), brought new elements to the study of androgenesis in rye. Applying appropriate stress conditions to the microspores is crucial for their switch to the sporophytic developmental pathway (Shariatpanahi et al., 2006). Different kinds of stress were required to induce androgenesis in certain species, although mainly a combination of heat and cold (Tenhola-Roininen et al., 2005) and recently osmotic stress were used in rye (Islam and Tuteja, 2012).

It is believed that the method of obtaining DHs through isolated microspore culture has several advantages compared to the anther culture (Oleszczuk et al., 2004). To date, the rare attempts of rye isolated microspore cultures have been mostly unsuccessful, usually resulting in no callus formation. So far only one group of researchers has reported full regeneration from isolated microspores of rye (Guo and Pulli, 2000). The authors suggest an important role of osmotic pressure of the medium, use of maltose as a carbon source and pre-treatment of anthers in mannitol solution. Optimized treatment conditions allowed the researchers to reach the regeneration efficiency of up to 6%. These results were partially confirmed in the next work of the same group (Ma et al., 2004), although this time the cold treatment proved to have the most significant impact on androgenesis performance, contradicting the earlier report.

One of the biggest obstacles in the production of a reasonable number of haploid lines is the loss of embryos and plants at various stages of *in vitro* culture. Only a small number of embryos developed in the culture is capable of germination. Most of them do not undergo conversion into plantlets and often form a secondary callus. Sometimes over 90% of germinating embryos develop into albino plants. Thus, the lack of chlorophyll in regenerated plants is considered to be a major problem in rye microspore embryogenesis (Immonen and Anttila, 1998; 1999). Albinism affects most of the *in vitro* cultured cereals: wheat (Liu et al., 2002), triticale (Oleszczuk et al., 2004), oats (Kiviharju et al., 2000), and barley (Makowska and Oleszczuk, 2014). Formerly, albinism was linked to the growth of temperature of the donor plant and subsequent culture conditions (Hoffmann and Wenzel, 1981). However, the actual causes of this phenomenon are yet to be recognized. 15–20% of green plants die after being transferred to soil. Of those that survive about 15–70% spontaneously double the number of chromosomes (Zimny, unpublished data), so usually, these plants are fertile. The rest requires additional treatment with toxic colchicine, which many plants do not survive.

Dubas et al. (2015) suggested that the low adrogenic potential in rye might be caused by its susceptibility to oxidative stress, which in turn decreases microspore viability. Similar observations were also reported in closely related triticale (Żur et al., 2019), as well as wheat (Sinha et al., 2016) and barley (Rodriguez-Serrano et al., 2012). The authors proved that one of major differences between responsive and recalcitrant triticale genotypes is the efficiency of antioxidative

defense. In responsive genotypes, such defense was stimulated directly by the cold treatment, which was not observed in recalcitrant genotypes. After application of antioxidant glutathione, higher microspore viability and embryogenesis rate were observed in recalcitrant genotypes. Other well known antioxidants were described as beneficial for androgenesis process in several grain species. Asif et al. (2013) reported that proline and glutathione supplementation increased embryo and green plant production in wheat and triticale, whereas salicylic acid reduced the number of albino plants. Ascorbic acid was observed to enhance embryogenesis and green plant regeneration in anther cultures of spring triticale (Yerzhebayeva et al., 2017). It is highly possible that the use of antioxidants in rye cultures might cause similar positive effects.

There are approaches other than anther and microspore cultures that enable haploid plants production. One of such methods is interspecific crossing, leading to the paternal chromosome elimination and formation of haploid embryo carrying maternal chromosomes only. Successful, yet labor-intensive, procedures were described for wheat and barley, with most commonly used pollen donors being maize and wild barley *Hordeum bulbosum* L. (Hayes et al., 2003; Niu et al., 2014). Recently, a similar attempt was made in rye (Marcińska et al., 2018). The researchers crossed 15 winter rye genotypes with maize and subsequently tested several factors possibly affecting haploid embryo formation. Out of over 17 thousand pollinated florets only 21 haploid embryos were recovered. Moreover, only six of the tested genotypes produced embryos, with more than half being produced by a single genotype. None of the obtained embryos was able to germinate. The results prove wide crossing to be particularly difficult and genotype-dependent in rye.

In the future, a promising alternative for rye wide crossing might be the use of haploid-inducer lines. Such lines, already described in *Arabidopsis* and sugar beet (*Beta vulgaris* L.), carry mutation in *CENH3* genes coding centromeric variants of H3 histones (Karimi-Ashtiyani et al., 2015). Upon selfing, such lines are stable, but when used in crosses they lead to haploid embryo development through paternal chromosome eliminations. Recently, it was demonstrated that CENH3-inducers can successfully produce haploids in maize (Kelliher et al., 2016), which proves the system to be valuable in at least one monocot species.

Despite many years of experiments, regeneration of DHs of rye is still a challenge and a strongly genotype-dependent process. Whereas in closely related species, great effort has been made on development of the highly effective methods of inducing androgenic callus and haploid embryos (Machczyńska et al., 2014; Machczyńska et al., 2015; Orłowska et al., 2016) in order to achieve plant cloning and genetic stabilization as well as in using gametoclonal variation in plant breeding.

GENETIC IMPACT ON CULTURE RESPONSE

In some monocot species, researchers managed to find a model genotype, e.g., in Potomac orchardgrass (*Dactylis glomerata* L.; Conger et al., 1983) and cultivars Igri (Hoekstra et al., 1992) or Bogo (Oleszczuk et al., 2004) in barley and triticale, respectively. With such a model rye genotype available, further research on the *in vitro* cultures could be carried out more easily, possibly giving reproducible results. To date, most research has been done using inbreed lines (Rakoczy-Trojanowska and Malepszy, 1993; Popelka and Altpeter, 2003). Among cultivars, Florida 401 (Guo and Pulli, 2000) and Auvinen (Ma et al., 2003) show relatively high regeneration potential.

Studies on the genetic determinants affecting rye *in vitro* regeneration are still not advanced enough (Targońska et al., 2013). Such limited knowledge causes the use of *in vitro* culture and genetic manipulation in rye breeding to be very difficult. Using wheat-rye chromosome substitution system, Pershina et al. (2003) associated the formation of embryogenic callus and overall morphogenetic capacity with the presence of unidentified genes located on chromosomes 2R and 3R. The same authors postulated suppression of the embryogenic callus formation to be associated with chromosomes 1R and 6R. Nine quantitative trait loci (QTLs) controlling somatic embryogenesis in immature embryos and inflorescences were mapped to rye chromosomes 1R, 4R, 5R, 6R and 7R, with some of them being a possible source of molecular markers for the selection of responsive genotypes (Bolibok et al., 2007). Studies conducted on triticale anther culture identified QTLs associated with androgenic response to be located on chromosome 4R, 5R and 7R (Krzewska et al., 2012). Gruszczyńska and Rakoczy-Trojanowska (2011) compared expression of four somatic embryogenesis-related genes in responsive and recalcitrant rye inbreed lines. The study indicated that the function of rye *Somatic Embryogenesis Receptor-like Kinase* (*ScSERK*), *Leafy Cotyledon 1* (*ScLEC1*), *Viviparous 1* (*ScVP1*) and ferredoxin-nitrite reductase (*ScNiR*) might be correlated with somatic embryogenesis. *ScNiR* expression seems to be positively correlated with regeneration capacity, whereas *ScLEC1* and *ScVP1* are probably negative regulators of somatic embryogenesis. *ScSERK* transcription is initially equal in both responsive and recalcitrant genotypes,

but at the subsequent induction and regeneration stages it seems to be higher in the recalcitrant line.

Highly efficient and genotype-independent regeneration techniques are available for such species as wheat and barley but not for rye (Eudes et al., 2003). Considering that fact, one possible way to achieve efficient regeneration in rye could be the use of novel methods successfully employed in other recalcitrant monocots. One of such methods, developed by DuPont Pioneer, employs an over-expression of morphogenesis-regulating genes to induce regeneration in recalcitrant maize genotypes (Lowe et al., 2016). The observation that ectopic expression of several specific genes might be sufficient to induce somatic embryogenesis was initially made in dicots (Zuo et al., 2002; Jha and Kumar, 2018). The combination of maize homologs of *Wuschel* (*WUS2*) and *Baby boom* (*BBM1*) turned out to be sufficient for high frequencies of somatic embryogenesis, not only in immature and mature embryos, but also in leaf explants. Lowe et al. (2016) used large cassettes containing resistance and reporter genes, as well as BBM1, WUS2 and CRE recombinase genes flanked with loxP sites. CRE expression under desiccation-induced promoter enabled excision of morphogenic regulators at the late stages of embryogenesis and subsequent regeneration of plants with normal phenotype. Interestingly, the same cassettes were later used in sorghum, sugarcane and rice, giving similar results (Lowe et al., 2016). Furthermore, the team developed a callus-free transformation-regeneration system based on *BBM1* and *WUS2* containing cassettes (Lowe et al., 2018). They were also able to eliminate the excision stage by using precisely selected non-constitutive promoters.

Khanday et al. (2018) described *BBM1* expression in rice (*Oryza sativa* L.) zygote, where *BBM1* is expressed directly after the fertilization from the paternal allele only, with maternal expression being initially blocked. Later, researchers proved that ectopic expression of *BBM1* under egg-cell-specific promoter leads to parthenogenesis. Such transformants were able to produce 5–10% haploid T1 seeds upon self pollination. Finally, combining egg-cell-specific over-expression of *BBM1* with MiMe background (a set of mutations replacing meiosis by mitosis) turned out to be sufficient for synthetic apomixis and high frequency of seed-propagated clone formation.

Morphogenic regulator-dependent techniques can also be easily combined with methods of vector delivery recently optimized for cereal microspores. Such morphogenic regulators could be delivered into rye microspores through electroporation (Bhowmik et al., 2018) or using cell penetrating peptides (Chugh et al., 2009) to facilitate efficient and genotype-independent production of DH plants.

Since 2016 a whole-genome draft sequence of rye has been available (Bauer et al., 2016), which undoubtedly will hasten genomic studies in rye.

CONCLUSIONS

Many attempts have been made to improve rye somatic embryogenesis and androgenesis induction in last few decades. Experiments clearly show that rye regeneration highly depends on the genotype used. An efficient regeneration method through somatic embryogenesis has been developed and successfully used for the genetic transformation in several rye genotypes. Such responsive lines, can now be used to identify molecular mechanisms determining regeneration efficiency in rye. More effort is needed to fully understand the regulation of morphogenic response and to develop methods for recalcitrant genotypes regeneration, similarly to the work done in maize (Lowe et al., 2016).

In Poland and other temperate climate European countries (e.g., Germany and Finland) there is a growing interest in embryogenesis from cells of the gametophytic pathway of rye. A highly efficient technique of DHs production is needed for hybrid breeding programs. For that reason, more basic research is required, answering the question of what causes the reprogramming of microspore development and how to achieve a large scale DHs regeneration. Advanced research on rye androgenesis is currently being conducted in Poland (Dubas et al., 2015; Zimny, 2018). It seems, however, that understanding of rye *in vitro* response is still incomplete. Due to limited success of traditional induction methods in rye cultures, new approaches are needed to support the existing techniques. Identification of rye homologs of genes already being used in other species to induce rapid cell divisions and regeneration should be the first step. Later, over-expression study in stable and transient systems might provide the foundation for such new approaches.

AUTHORS' CONTRIBUTIONS

KM and JZ took responsibility for the integrity of the work as a whole. Authors declare no conflict of interest.

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