BioTechnologia • Journal of Biotechnology, Computational Biology and Bionanotechnology • Vol. 96(2) 2015

BioTechnologia

Journal of Biotechnology, Computational Biology and Bionanotechnology

QUARTERLY Volume 96(2) • 2015

optimization of *in vitro* culture
EU law on GMOs

XIV Overall Polish
in vitro Culture
and Plant Biotechnology
Conference

September 14-17, 2015
Poznań

Committee of Biotechnology • Polish Academy of Sciences
Institute of Bioorganic Chemistry • Polish Academy of Sciences

Journal of Biotechnology
Computational Biology
and Bionanotechnology

http://www.biotechnologia-journal.org
e-mail: office@bta-journal.org

BioTechnologia

Volume 96(2) • 2015

ISSN 0860-7796

Publishers

Committee of Biotechnology, Polish Academy of Sciences
ul. Noskowskiego 12/14, 61-704 Poznań
http://www.kbiotech.pan.pl, e-mail: twardows@ibch.poznan.pl

Institute of Bioorganic Chemistry, Polish Academy of Sciences
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Front cover: Somatic embryos of the tree fern *Cyathea delgadii* Strenb by Anna Mikuła

Printed in Poland
(Moś & Łuczak, Poznań)

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BioTechnologia vol. 96(2) • pp. 161-170 • 2015
Journal of Biotechnology, Computational Biology and Bionanotechnology OPINION

Recent changes to EU law on GMOs and their potential influence on the patentability of GM plants. Some remarks on possible side effects of Directive 2015/412/EU

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Abstract

In this article I present recent changes in EU legislation on the cultivation of GM plants and I attempt to answer the question as to whether the new laws continue to follow the precautionary principle approach and the case by case approach that characterized the European Union's GMO legislation until recently. Also, given the nature of the newly introduced grounds for restricting the cultivation of GMOs, I try to find out if the new legislation could influence the patentability of transgenic plants or methods of their production. While growing in popularity around the world, transgenic plants face strong opposition within the European Union. Recent changes to EU legislation governing the cultivation of GM plants are just another example of the said opposition. Directive (EU) 2015/412 of the European Parliament and of the Council amending Directive 2001/18/EC provided member states with means to restrict or effectively prohibit cultivation of genetically engineered plants in their territories, even if such plants have already been authorized for cultivation in the EU. The reasons countries can currently invoke in order to introduce limitations are no longer restricted to bio-safety, but rather encompass a set of political and social issues such as socioeconomic impacts, avoidance of GMO presence in other products, agricultural policy objectives, public policy etc. They are to a much lesser extent (than up till now) based on the precautionary principle, as possible restrictions will also concern already examined and authorized GMOs. Restrictions no longer need to target particular transformation events, they can now encompass certain traits or crops. When it comes to the patentability of GM plants or methods of their production, the recent changes seem to have limited influence, given the European Patent Office's stance on the application of morality and "ordre public" exclusions and its relative independence from EU law. The possibility cannot be excluded that local laws adopted on the basis of the newly introduced changes could influence procedures before local patent office, should those offices decide to apply the morality or "ordre public" exception to patentability. The newly adopted laws have a rather different effect, though. The profitability of developing GM plants in the EU (and their patenting) may become questionable, should the exploitation of such inventions be prohibited in several EU member states.

Key words: GMO, european law, precautionary principle, patent law, patentability

Introduction

The European Union has some of the strictest laws regulating the use of genetically modified organisms (Davidson, 2010). Nearly every activity involving such organisms – from research to marketing and post marketing monitoring – is regulated by EU legislation. Stringent regulations apply in particular to placing genetically engineered products on the market, not only as food or feed but also as sowing material. Under the European Union's regulations, planting a GM crop is only allowed if that particular crop has passed a thorough risk assessment procedure and has been approved for cultivation by either a competent authority in one of the member states or by the Commission of the European Union. So far, only MON 810 corn has been approved for cultivation and this is grown in Spain, Portugal, the Czech Republic, Romania and Slovakia (James, 2015).

This article presents recent changes to EU legislation on the cultivation of GM plants and compares them to previous rules, in order to test a hypothesis that the recently introduced changes constitute a departure from the precautionary and case by case approaches that have thus far been fundamental. The article also examines whether the new legislation could influence the patentability of GM plants, given that the new reasons for restricting the use of GMOs are based on political and

axiological concerns, which also constitute reasons for excluding the patentability of certain inventions. Both issues are examined via a comparison of various legal acts and also an interpretation of provisions in the context of past and current case law.

Use of GM plants in agriculture is regulated by *Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC* ("Directive 2001/18/EC"). The objective of the Directive is, "in accordance with the precautionary principle, to approximate laws in the area as well as the laws, regulations and administrative provisions of the member states, and to protect human health and the environment when (...) placing on the market genetically modified organisms as or in products within the (Union)". The directive mandates that member states adopt legislation providing that any person planning either to deliberately release GMOs into the environment for experimental purposes or to market them as products, does so only after performing proper risk assessment and obtaining authorization from competent authorities. There is a zero tolerance threshold for placing on the market GMOs that have not been authorized for marketing according to EU law (see, the European Court of Justice Judgment in case C-442/09).

The legislation (at least until quite recently – see below) was based on the precautionary principle (PP), which is a procedural (decision making) rule used in risk management and risk analysis. The principle mandates that decision makers should undertake risk limiting measures "where scientific evidence is insufficient, inconclusive or uncertain and there are indications through a preliminary objective scientific evaluation that there are reasonable grounds for concern that the potentially dangerous effects on the environment, human, animal or plant health may be inconsistent with the chosen level of protection" (COM 2000 (1)). The measures taken have to be proportional to the chosen level of protection, non-discriminatory in their application, consistent with similar measures already taken, based on an examination of the potential benefits and costs of action or lack of action, subject to review in the light of new scientific data, and capable of assigning responsibility for producing the scientific evidence necessary for a more comprehensive risk assessment (COM 2000 (1)). In the legislation concerning the use of GMOs, PP is manifested in the requirement that all GMOs planned for marketing should be thoroughly examined and undergo risk assessment before they reach the common market. Only if the effects of the said assessment are favorable (i.e. cultivation and consumption do not pose any threats to human or animal health or to the environment) can a particular GMO be authorized for marketing. While criticized at times for its low cost effectiveness and vagueness (Jansen van Rijssen, Eloff, Morris, 2015), PP constitutes the cornerstone of the EU's approach to GMOs.

Another important principle strongly connected to PP is one that mandates acting according to a case by case approach. According to this principle, all GMOs (all transformation events) should be treated individually, so that no general conclusions are drawn as to their safety as products. Consequently, for instance, the case by case approach does not permit the assumption in the decision making process that GM plants are generally safe for consumers or that they are harmful, based solely on experiences with different GMOs. the potential effects of growing e.g. herbicide resistant corn and Bt corn may differ. Such effects depend on a plethora of factors: the function of the insert, the species, its ability to reproduce and survive in the environment, the vector used etc. The presented approach does not allow the formulation of general conclusions about the safety of broad groups of organisms (e.g. all GMOs, GMOs of a particular species or GMOs possessing a particular trait). The application of both PP and the case by case approach bears significant costs.

PP is designed for decision making in situations of significant scientific uncertainty. Its application is costly and requires measures, which may prove unnecessary (Gadomski and Zimny, 2009). Also, the cost of delaying an action because of the precautionary principle, and hence losing benefits that may possibly stem from the said action, has to be factored into the general costs of PP application (Cantley, 2012). The scope of PP application should be limited to situations where there is not enough scientific data to support a claim that a certain action is either safe or too risky. In such cases, other risk management strategies should be applied.

Therefore until recently, after a particular GMO had been approved for marketing in the European Union, member states were not allowed to introduce restrictions on products containing that GMO or the GMO itself. According to art. 22 of Directive 2001/18/EC,

"Member States may not prohibit, restrict or impede the placing on the market of GMOs, as or in products, which comply with the requirements of this Directive". However, the next article of the same Directive contains a safeguard clause, according to which member states can provisionally restrict or prohibit the use or sale of such authorized GMOs as or in a product on their territories. There are several conditions that have to be met in order to impose such restrictions legally. Firstly, they have to target particular GMOs, so the safeguard clause cannot be invoked in order to restrict the use of GMOs in general or those belonging to a particular species, etc. Secondly, the safeguard clause may only be invoked when there are reasons to believe that a previously authorized GMO constitutes a risk to human health or the environment. This belief has to be based on detailed grounds stemming from new or additional information made available after the date of the authorization or from reassessment of the existing information on the basis of new or additional scientific knowledge. Restrictive measures should thus be based on some new scientific information, according to which the risk assessment that was carried out before the authorization, did not reveal potential threats connected with the use of the authorized GMO. It is a duty of the member states to introduce and enforce efficient laws with the aim of preventing serious harm to the environment (Nanda et al., 2013). Political or social reasons, such as public aversion towards GMOs, political statements declaring countries or their parts "GMO free", cannot constitute grounds for applying the safeguard clause from art. 23 of Directive 2001/18/EC, or adopting restrictive measures. However, the above clause has been invoked in the past by several countries aiming to restrict the use of some GMO products in their territory, contrary to the scientific opinions of such bodies as the European Food Safety Authority (EFSA) or their local scientific committees (Davidson 2010)[1]. On some occasions, certain restrictive measures were subsequently approved by the Council. Pursuant to directives regulating plant breeding, it is also possible to restrict the use of seeds of particular varieties. In spite of this fact, until recently, member states had limited possibilities to impose restrictions on already authorized GM products. This situation has changed significantly after the adoption of Directive 2015/412/EC, which added some crucial provisions to Directive 2001/18/EC.

Recent changes in EU laws on the cultivation of GM plants[2]

Following the Commission's recommendation of 2010 (*Recommendation on the guidelines for the development of national co-existence measures to avoid the unintended presence of GMOs in conventional and organic crops*), which proposed the introduction of the possibility for member states to opt out of the cultivation of GM crops, the European Parliament and Council adopted Directive 2015/412/EC, *amending Directive 2001/18/EC as regards the possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory*. Firstly, the Directive imposes a new obligation on those member states where GMOs are grown. As from 3 of April 2017, those states "shall take appropriate measures in border areas of their territory with the aim of avoiding possible cross-border contamination into neighboring Member States in which the cultivation of those GMOs is prohibited, unless such measures are unnecessary in the light of particular geographical conditions". This provision could be of significance for the Polish case, since the cul-

[1] It is clear that the safeguard clause – initially designed to protect consumer health in urgent cases – has been used as a means to eliminate GM plants from particular countries' agriculture. It was an efficient method, given the limited number of transgenic plants approved for cultivation (practically only one). Some countries resorted to slightly different methods. A notable example of this is the Polish Council of Ministers' *Regulation on the prohibition of use of the sowing material of MON 810 corn varieties*, which prohibits cultivation of over 200 MON 810 varieties. The regulation is based on a provision of the *Act on seed production*. Allegedly based on some safety concerns, this regulation leads to a paradox, where it is legal to use MON 810 corn as food, feed or even to sell its sowing material, but it is illegal to grow it. It may be that the reason for the adoption of Directive 2015/412/EC was to allow member states to effectively remove GM plants from their landscapes, without them having to resort to such convoluted schemes as that presented above.

[2] It is worth mentioning that the Polish *Law on GMOs* also underwent important changes in early 2015, with the adoption of an act introducing serious changes to the existing regulations (Dz. U. 2015.277). Important as they may be, the changes mostly affect contained use of GMOs and GMMs (where the changes liberalize the administrative procedures somewhat, yet not to the extent that is permitted in EU law) and deliberate release for experimental purposes. New changes to Polish law have been based on earlier EU legislation and do not have EU-wide effect and mostly affect the scientific community. Therefore, they fall outside the scope of this article and have been, as such, deliberately omitted.

tivation of MON 810 is currently prohibited in Poland, while two of the countries where the corn is currently grown in the EU (Slovakia and the Czech Republic) border Poland. The provision may be of lower significance in this case, since the border passes mostly through mountainous areas, limiting the possibility of pollen flow or other forms of trans-border movement. Were some GM plants grown in eastern Germany for instance, the importance of the cited provision could rise.

The most striking change introduced in Directive 2015/412/EC is the one allowing member states to restrict the use of GMOs in their territory for reasons other than those connected with human, animal or environmental safety. According to the newly introduced article 26b, even before a new GMO has been authorized for marketing (during the authorization procedure) or when an authorization is renewed, a member state may demand that the geographical scope of authorization or consent be so adjusted that the territory of that member state or its part are excluded from that authorization. This restriction can apply not only to the consent for cultivation, but also to decisions authorizing GMOs as food or feed (art. 26b of Directive 2001/18/EC). Effectively, this means that even before the GMO is authorized, member states can opt out from its use in their territory.

Even if a member state did not request a geographical restriction initially, it is still entitled to introduce restrictive measures (be it because of a change in the government or a change of local policies etc.). The adopted measures can mean the restriction or even prohibition of cultivation in all or part of the territory of the member state. Restrictions can target not only particular GMOs but also groups of them, defined by crop or trait (e.g. "transgenic corn", "GMOs with herbicide resistance" and combinations thereof). Such restrictions have to conform with European Union law, and be reasoned, proportional and non-discriminatory (for instance, they cannot target GMOs basing on their country of origin or a manufacturer). A country aiming to introduce such post-authorization restrictions has to base them on one or more of the following reasons:

a) environmental policy objectives;
b) town and country planning;
c) land use;
d) socioeconomic impact;
e) avoidance of GMO presence in other products (...);
f) agricultural policy objectives;
g) public policy.

The above grounds can be invoked individually or in combinations, with the exception of "public policy", which cannot be invoked individually. Invoked grounds cannot conflict with the environmental risk assessment carried out in order to authorize the GMO as food, feed or sowing material. This means that states wanting to introduce bans on GMOs cannot simply claim that they have reservations as to the safety of their use, without having scientific reasons for it. Should they have such reasons based on scientific knowledge, they ought to apply the safety clause as mentioned earlier. This limitation of the possibility to introduce a restriction or prohibition may prove to be purely hypothetical, as most of the grounds countries can invoke are detached from issues of biosafety, which are addressed in the risk assessment procedure. While some environmental safety concerns can be deduced from the first prerequisite (environmental policy objectives), they are not necessarily present in several others, such as socioeconomic impact, land use or public policies. The Commission can comment on proposed measures; its comments are nonbinding, however.

The newly adopted legislation constitutes a radical departure from the major principles that constituted the basis of the previous legal order. While the authors of the new directive seem to have invoked the Precautionary Principle as one of the grounds for its adoption (see Recital 2 of Directive 2015/412/EC), it is quite difficult to view new provisions as being particularly representative of the said principle. PP justifies application of restrictive measures if accessible scientific data do not suffice to rule out or at least efficiently manage the apparent risks connected with some action, be it growing GMOs or any other. Meanwhile, new provisions allow countries to prohibit cultivation of some GMOs even though those GMOs have passed the risk assessment procedure. A prohibition based on reasons unconnected with biological safety issues or on reasons that seem to be of ideological nature cannot be seen as an example of applying the Precautionary Principle. Irrespective of the fact that PP was differently defined in the past (see COMEST 2005, p. 13), there are some common elements of its application, including: scientific analysis, and the existence of considerable scientific uncertainties as to causality, magnitude, probability and the nature of

harm. The interventions should be proportional to the chosen level of protection and the magnitude of a possible harm (COMEST 2005 p. 13-14).

Even if we were to assume that the new legislation aims at constraining or containing possible harm stemming from the mere fact that transgenic plants are grown in a particular member state and even if we were to assume that that possible harm may occur in spheres other than biological safety (e.g. local economy, spatial planning etc.), it is still quite difficult to view the new restrictions as an application of PP. Not only is the plausibility of the occurrence of such harm not too well assessable, but also such harm is difficult to define when it comes to its magnitude and extent. Hence, it is also dubious that such measures are proportional to the possible harm.

Another significant change brought about by the new legislation is a serious limitation of the case by case principle. Unlike in the case of the safety clause, where restrictive measures are supposed to target particular GMOs, restrictions adopted according to the new laws can apply to broader groups of transgenic organisms. According to new art. 26b par. 3 of Directive 2001/18/WE, "a Member State may adopt measures restricting or prohibiting the cultivation in all or part of its territory of a GMO, or of a group of GMOs defined by crop or trait". An application of the case by case principle in decision making about the marketability of GMOs is justified by the nature of genetic modification. Since there are multiple ways of modifying multiple features of an organism, there are also multiple issues to be addressed when it comes to risks connected with those procedures. "The objective of an environmental risk assessment is, on a case by case basis, to identify and evaluate potential adverse effects of the GMO, direct and indirect, immediate or delayed, on human health and the environment which the deliberate release or the placing on the market of GMOs may have. The environmental risk assessment should be conducted with a view to identifying if there is a need for risk management and if so, the most appropriate methods to be used." (Directive 2001/18/EC Annex II p. A) In order to achieve that goal (a) risk assessment has to be carried out on a case by case basis, in a scientifically sound and transparent manner based on available scientific and technical data. The identified characteristics of the GMO and its use which have the potential to cause adverse effects should be compared to those presented by the non-modified organism from which it is derived and to its use under corresponding situations (Directive 2001/18/EC Annex II p. A and B). Amongst the traits tested in higher plants, these include the likelihood for the GMO to become persistent and invasive, the potential for gene transfer to other species, potential environmental impacts on target and non-target organisms, etc. The features of the insert and vector are also taken into account (Directive 2001/18/EC Annex II, III). Identification of these traits can constitute a basis for deciding whether a particular organism is safe to be released into the environment or not. All the above mentioned factors justify the case by case approach, since a change in one of them can have serious safety consequences.

The newly adopted rules allow individual traits of GMOs in question to be completely disregarded, as the restrictions may apply to whole groups of organisms, defined solely by their trait or crop. This means that member states can introduce restrictions on the cultivation of e.g. transgenic corn, Bt corn, etc. This fact, combined with the recently adopted grounds for justifying the restrictions that are, for the large part, not connected to biological or environmental safety issues, marks a radical change in the EU's approach to GMOs.

The obstacles to placing such organisms on the market, which were amongst the largest in the world (Davidson, 2010), have now become even more daunting. This may impact not only upon entrepreneurs interested in marketing GM plants in the EU, but also upon farmers and scientists. It is, however, too early to estimate the extent and seriousness of these impacts. There is a group of possible impacts that seem less obvious, though. They pertain to the influence of the recent changes on the patentability of GM plants.

Possible impacts on the patentability of GM plants

Despite a lengthy and vigorous debate about the patentability[3] of GM plants or the patentability of living organisms in general (see Crespi, 2000), genetically modified plants and processes of their making are currently pa-

[3] Patentability can be defined as a set of features, such as novelty, inventive step and industrial applicability, which an invention has to possess to be protected by a patent. There are also factors limiting patentability, in particular those connected with the moral and legal aspects of the exploitation of an invention.

tentable. According to art. 3 of Directive 98/44/EC *on the legal protection of biotechnological inventions* (henceforth Directive 98/44/EC), "inventions which are new, which involve an inventive step and which are susceptible of industrial application shall be patentable even if they concern a product consisting of or containing biological material or a process by means of which biological material is produced, processed or used". Also, "biological material which is isolated from its natural environment or produced by means of a technical process may be the subject of an invention even if it previously occurred in nature". According to art. 4 par. 1 let. a, plant and animal varieties are excluded from patentability; however, this exclusion does not preclude patenting of transgenic plants in general (Stercx and Cockbain, 2012). As stated in Recital 31 to Directive 98/44/EC, "a plant grouping which is characterized by a particular gene (and not its whole genome) is not covered by the protection of new varieties and is therefore not excluded from patentability even if it comprises new varieties of plants". Hence, it is possible to patent e.g. a transgenic, herbicide resistant corn, where the resistance is conferred by a particular introduced gene or set of genes, so long as the patent claims do not refer to a particular variety of that corn. This issue is currently considered rather controversial in the doctrine (see Sterckx and Cockbain 2012 p. 193-242).

There are other reasons for excluding the patentability of certain inventions, namely those, whose commercial exploitation would be contrary to morality or "ordre public". This exclusion was introduced in art. 6 par. 1 of Directive 98/44/EC, but it was in one way or another a part of the patent law even before the adoption of that directive. In particular, according to art. 53 (a) of the European Patent Convention (henceforth EPC), "inventions, the commercial exploitation of which would be contrary to "ordre public" or morality; such exploitation shall not be deemed to be so contrary merely because it is prohibited by law or regulation in some or all of the Contracting States". Such exclusions are also present in Polish patent law (see art. 29 par. 1 pt. 1 and art. 93^3 of *Industrial Property Law* of 2000). Since its inclusion in the patent law, this exclusion from patentability has been used rather rarely. Its "renaissance" began with the advent of biotechnological inventions. One of the reasons for this was the fact that such inventions often touch spheres that are of particular importance, such as environmental safety, human dignity, autonomy etc. Another reason for the increased importance of the "morality exclusion" was that various groups that opposed the granting of patents on living organisms and biotechnological inventions in general, invoked this exclusion in order to prevent the granting of such patents. One of the more significant cases brought before the Technical Board of Appeal of the European Patent Office (henceforth EPO) was Case T 356/93 (Plant cells), where the applicant sought to receive a patent for herbicide resistant plants, cells of such plants and methods of their production. An opposition against the granting of said patent was filed by Greenpeace Ltd. on, among others, the grounds that the granting of a patent for plant life forms and the exploitation of the patent was contrary to morality or "ordre public" (Decision T 356/93 par. II). Not only was the mere fact that plants are patented questioned on moral grounds, but also safety concerns connected with the possible effect of such plants' release into the environment were raised. The Technical Board of Appeal was tasked with defining the terms "morality" and "ordre public" for the purposes of patent law, as well as with deciding whether moral or legal reasons justified revocation of the granted patent. In its decision, the board stated that "the concept of morality is related to the belief that some behavior is right and acceptable whereas other behavior is wrong, this belief being founded on the totality of the accepted norms which are deeply rooted in a particular culture. For the purposes of the EPC, the culture in question is the culture inherent in European society and civilization. Accordingly, under Article 53(a) EPC, inventions the exploitation of which is not in conformity with the conventionally-accepted standards of conduct pertaining to this culture are to be excluded from patentability as being contrary to morality". Also, the board decided to define the concept of "ordre public", by stating that it "covers the protection of public security and the physical integrity of individuals as part of society. This concept encompasses the protection of the environment as well. Accordingly, under Article 53(a) EPC, inventions the exploitation of which is likely to breach public peace or social order (for example, through acts of terrorism) or to seriously prejudice the environment are to be excluded from patentability as being contrary to "ordre public".

Setting aside several problems inherent in these definitions, in particular the rather blurry concept of "cul-

ture inherent in European society and civilization", as a basis for the formation of moral norms according to which the inventions should be measured or the concept of one public order in all contracting states (Hansen, 2002), the presented definitions indicate some important areas where the patentability of transgenic plants could be questioned. Chiefly, inventions whose exploitation would seriously endanger the environment would be considered not patentable on the grounds of their exploitation being contrary to the "ordre public". While the exploitation of an invention can only be considered as offending the "ordre public", if it is already prohibited by law (Schatz, 2000). This is the case when it comes to most GM plants, because their patentability is usually examined before they are authorized for marketing. It should also be noted that the exploitation of an invention that is harmful to the environment would also most likely be deemed contrary to morality. The question remains, however, if the patent office should assume that a yet unauthorized GM plant threatens the environment, or should such an office give the plant the benefit of the doubt. The EPO Technical Board of Appeals settled for the latter option. In the already cited decision, T 356/93/EC, the board stated that "it would be unjustified to deny a patent under Article 53(a) EPC merely on the basis of possible, not yet conclusively-documented hazards (...) Should the competent authorities and bodies, after having definitively assessed the risks involved, prohibit the exploitation of the invention, the patented subject-matter could not be exploited anyhow. If, however, regulatory approval is given based on the finding that no risks or minimal risks are involved, then patent protection should be available". As was further stressed, patents should not be granted for inventions that would relate to misuse or a destructive use of plant biotechnology (T 356/93, p. 17.1). Examples of these would include plants specially designed to cause harmful effects, plants used as biological weapons etc. This is not the case in respect to most, if not all currently developed transgenic plants that are being patented with the intention of their subsequent marketing. Since the purpose of their development is to improve some agronomical, dietary or other trait, it is the risks connected with their exploitation that could bar the possibility of patenting, not their purpose or the method by which they were created (genetic modification). In order to deny or revoke a patent, the patent office should be presented with reliable information that the exploitation of that particular invention would seriously prejudice the environment. As the board stated, "the revocation of a European patent under Article 53(a) EPC on the grounds that the exploitation of the invention for which the patent has been granted would seriously prejudice the environment presupposes that the threat to the environment be sufficiently substantiated at the time the decision to revoke the patent is taken by the EPO" (T 356/93, p. 18.5). This stance was also maintained in other cases brought before the EPO Technical Board of Appeals (see e.g. case T 179/01 par. 7) Hence, in the case of patenting transgenic plants the situation seems quite different than in the case of their authorization for marketing. In the latter case, it is impossible to obtain an authorization until the plant in question is proven to be safe. In the case of their patentability, a patent can be denied or revoked on the basis of morality or "ordre public", if the patent office decides that it was sufficiently proven that the invention in question will seriously harm the environment or pose other kinds of threats. This approach seems reasonable, since denying patents on the basis of some general presumptions or hypothetical threats could practically preclude patenting not only transgenic organisms but also medicines and other products that require some form of authorization before they are placed on the market.

The newly adopted laws regarding the cultivation of GM plants may raise questions as to their influence on their patentability. As mentioned earlier, the new reasons for restricting cultivation often have little to do with biological safety issues. Rather, they address other types of concerns, such as the possible influence of GM crop cultivation on the local social or economic situation, the relation of such cultivation to however defined agricultural policy objectives, town and country planning etc. These are issues of some moral importance. A question thus arises as to whether a patent office could deny a patent on the grounds of morality or "ordre public", based on the fact that local policies prohibit the use of GMOs belonging to the species of the patented invention, or laws prohibiting the use of GMOs possessing a trait that is also characteristic for the patented invention (viz. a transgenic plant). It seems that this would be quite unlikely in the case of proceedings before the European Patent Office. Firstly, as has already been mentioned, article 53 (a) of the EPC states that the of-

fice cannot simply deem exploitation of an invention contrary to morality or the "ordre public", based merely on the fact that such exploitation is prohibited by law or by a regulation in some or all of the contracting states. One could argue that any inconsistency with morality may lie deeper and not follow simply from the fact that an anti-GMO policy was adopted in a particular state, but from the fact that this policy protects some important values. It would then be those values which had been threatened by the exploitation of the invention. In such a case, the denial or revocation of a patent could be justified not by a mere contradiction to local laws, but by the influence of the invention on some spheres of human activity that are particularly valued. Such argumentation would not hold either, and there are several reasons for this.

Firstly, the European Patent Convention is a standalone international agreement and, despite the fact that all the members of the EU are parties to the convention, it itself is not a part of EU law. There are currently 38 members of the European Patent Organization, only 28 of them are members of the EU. Thus, the EPO is not an institution of the EU and is not bound by its laws. There is some form of indirect influence of both legal orders, e.g. the substantial provisions of Directive 98/44/EC have been added to the Implementing Regulations of the European Patent Convention in order to harmonize it with laws adopted in EU member states. The European Patent Office, not being an EU institution, is not accountable to any judicial body of that organization, including the European Court of Justice[4] and has refused to refer legal questions to that court in the past (see e.g. Decision G 2/06). A change in EU laws concerning member states' ability to prohibit the cultivation of some GM crops should not be considered an obstacle to granting a patent for some transgenic plant or method of its production. Besides, it is highly unlikely that restrictions or prohibitions based on the new laws would be introduced in all the EU member states, considering the fact that GM plants are currently grown in some of those states.

The argument that there are local policies prohibiting the cultivation of some GM crops and that the exploitation of a GM plant would violate those policies or important values protected by them could be used in procedures before local patent offices. The patent office would then have to cope with the local legal situation and issues of moral importance in the territory of a particular state. Such a belief should be opposed. Even though there may be some moral justification underlying the introduction of restrictions on growing certain GMOs, those arguments do not automatically render the exploitation of an invention immoral. This issue should be considered by the patent office on a case by case basis and yet it still seems dubious that an invention could be considered immoral, because its exploitation was prohibited based on socioeconomic factors or, e.g., town and country planning. Also, the exploitation of such an invention should not be considered immoral, because of the fact that GMOs or their patenting are unpopular in a particular state (see Tosun, 2014). As has been mentioned several times by the EPO (see e. g. decisions T 356/93 p. 15, T 315/03 p. 10.4), public opinion polls are not suitable for deciding on what is morally acceptable or not, as their results can be easily skewed. Besides, the opinion of the majority does not necessarily reflect what constitutes part of morality or "ordre public". To sum up, while it cannot be excluded that there may be attempts to prevent the granting or revoking patents on GM plants, based to some extent on the newly adopted

[4] This does not mean that the European Court of Justice (ECJ) does not influence patent law at all. Several of its judgments touch upon the subject. Notable examples include: case C-377/98, where the court decided that Directive 98/44/EC was in conformity with EU law or case C-34/10, where the court, on moral and "ordre public" grounds, excluded the patentability of certain types of human embryonic stem cells and methods of their procurement and also defined the term "human embryo" for the purposes of patent law. Another example is case C-428/08, where the ECJ ruled that a patent protecting a DNA sequence is extended to biological material in which the genetic information is contained and performs its function (e.g. sowing material). This protection does not cover products, where the patented sequence does not perform its function anymore, for example soy meal. In the discussed judgment a so-called "absolute protection" of DNA sequences was also ruled out. Important as they might be, the presented judgments are of limited significance from the point of view of the goals of this article. In particular, case C-34/10 tackles the issues of morality and "ordre public" as grounds for excluding patentability of inventions, but it does so in the field of bio-medical research, where human biological material is used. Case C-428/08 applies to patenting GM plants and DNA sequences directly, but focuses more on the scope of patent protection and does not discuss issues of moral matter. A possible situation, where case C-428/08 would apply to our considerations would have to include an attempt to patent a DNA sequence, whose sole function was in itself incompatible with morality or "ordre public". The patent would not be granted, since the scope of protection would be limited to that function, but morality or public order exception would preclude patent protection of that very function. Such a situation does not seem likely, however.

GMO legislation, it would constitute a radical change of currently established approach to that matter, if the patent offices decided to do so.

A different type of issue should also be considered here. Instead of debating whether obtaining a patent for transgenic plants in Europe is still possible, one should perhaps estimate whether it is economically justifiable (see also Cantley, 2012). Obtaining a new GM plant requires serious investment both monetary, and in terms of time and human resources. Meeting the criteria for authorization is also quite costly and time consuming, given how thorough the safety requirements and the risk assessment procedures are. Also, obtaining and maintaining a patent can prove quite costly, if one considers not only the cost of procedures and patent protection fees but also the costs of representation, translation etc. (although this would constitute only a fraction of the earlier mentioned costs). Ultimately, the patent holder may still be unable to market their product legally in some of the member states, due to the local restrictions on the cultivation of GMOs. It can be argued that the new developments in terms of EU laws on GMOs, while not impacting directly upon their patentability, provide for a hostile environment for the exploitation of such inventions.

Conclusions

The recent developments of laws on the cultivation of GM crops allow member states to restrict the use of such crops for reasons other than ones connected with bio-safety. This new approach constitutes a departure from the earlier policies, based on the precautionary and case by case approaches. Under the new legislation, member states can prohibit the cultivation of whole groups of GMOs in their territories, based on the requirements of local policies or social and economic concerns. This radical change in the approach could hypothetically influence the patentability of GM plants or methods of their production. This, however, seems rather unlikely, given the way the European Patent Office interprets the exclusions from patentability that are based on the grounds of morality or "ordre public". The possibility cannot be excluded that there may be attempts to invoke local policies prohibiting the use of GMOs in individual countries on the grounds of a denial or revocation of patents before local patent offices. Such attempts should be met with opposition. The mere fact that the cultivation of a GM crop is prohibited by law should not mean that the invention cannot be exploited in a different way or that the cultivation of such a crop is to be considered immoral. The new laws make the already unfriendly legal environment surrounding GMOs in the EU quite hostile, rendering the economic viability of such undertakings as GMP development, patenting and marketing, questionable.

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BioTechnologia vol. 96(2) • pp. 171-177 • 2015
Journal of Biotechnology, Computational Biology and Bionanotechnology REVIEW PAPER

Assessment of winter oilseed rape DH lines using uni- and multivariate methods of quantitative genetics and mathematical methods

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Abstract

The development and improvement of an *in vitro* androgenesis of oilseed rape has allowed to obtain doubled haploid (DH) populations on a large scale. It has become possible to use DH lines in a genetic analysis of quantitative traits. Among the other benefits of DHs, homozygous state allowed to use them in a series of experiments aiming at the efficient selection of desired genotypes and their examination for adaptation in various environments. This is a review of our earlier studies which have confirmed numerous possibilities of using winter oilseed rape DH lines in a statistical analysis. Populations of DHs were used for: estimation of genetic parameters, transgression effects as well as a general and specific combining ability, genetic variation in DH populations or effects of the cross direction. In this paper, possibilities of a comprehensive multidirectional assessment of DHs lines of oilseed rape through the application of uni- and multivariate methods of quantitative genetics and mathematical methods are also presented.

Keywords: *Brassica napus* L., doubled haploids, uni- and multivariate methods, transgression, genetic variation

Introduction

The development and improvement of the process of the *in vitro* androgenesis of oilseed rape enables doubled haploid (DH) populations to be obtained on a larger scale, facilitating both the application of DH lines in the genetic analysis of quantitative traits and the research into the impact of the environment on the yield and quality of the seed. Because of its homozygosity, a single DH line produces only one type of gamete which, in controlled conditions, duplicates its own genotype. This allows to perform multiple experiments with the same genotype, in various locations and different years, which is not possible with a generation of segregated hybrids in classical breeding. Moreover, for traits controlled by polygenic inheritance, the DH technology requires fewer genotypes, because there are no heterozygotes; thus, no dominance effects, and no interactions of non-allelic heterozygous loci can be observed.

Estimation of genetic parameters

The knowledge of effects of expression of genes controlling quantitative traits enables to choose the strategy of breeding. One of the methods to obtain this information is the estimation of the genetic parameters.

A series of field experiments were carried out on 32 DH lines of winter oilseed rape, their parental forms (DH C-1041, DH O-120) and F2 and F3 hybrids to evaluate genetic parameters controlling yield components. Such parameters may include the oil content and fatty acids in oil, the number of genes or closely linked gene clusters needed to control the content of these acids. Based on the DH lines and respective segregating generations, the genetic parameters determining the effects of the additive gene action, domination and the non-allelic interaction of homozygous and heterozygous loci were estimated for the length of pods, the number of seeds per pod, 1000 seeds weight and the oil content (Adamska et al., 2002). The effects of the additive gene action were significant for each studied trait. The dominance was significant only for the length of pod and the number of seeds per pod. The effect of heterozygous gene action was not observed in this study. Genetic parameters for individual fatty acids content in oil were described by Cegielska-Taras et al. (2005).The effects of the additive gene action calculated for every year separately and

jointly for three years were significant for all the analyzed fatty acids. The effects of the interaction between the homozygous loci positively influenced the increase in the oleic acid content. The dominance effects were observed from a three-year experiment, only for the palmitic acid (Cegielska-Taras et al., 2005). The effects of non-allelic interactions of heterozygous loci were insignificant for the content of all studied acids in all years.

Estimation of transgression effects

Due to their homozygosity, DH lines are excellent material for the investigation of the occurrence of transgression effects. The most common explanation of that phenomenon is that transgression is the result of a complementary gene action. The frequency of occurrence of transgressive genotypes depends, above all, on the genetic differentiation of parental components and their phenotypic similarity. In homozygous populations, the lines that are significantly better than the higher-scoring parent, or significantly worse than the lower-scoring parent in respect of a given trait are considered transgressive (Kuczyńska et al., 2007). In the case of a great number of DH line populations and a small number of seeds only an unreplicated experiment with a replicated standard gives a possibility to estimate all genotypes. Kaczmarek et al. (2009) proposed statistical methods for both a phenotypic and genetic analysis and the estimation of the transgression effects of winter oilseed DH lines evaluated in unreplicated experiments. The statistical methods were described based on the example of the data obtained for fat content and for the thousand seed weight from an unreplicated experiment including 210 DH lines with parental forms as standards. The application of these methods, especially the method of contrasts, enabled selection of transgressive DH lines by subsequent unreplicated experiments carried out with regularly distributed standards – two parental forms.

Two populations of DH lines of winter oilseed rape obtained from F1 hybrids from the reciprocal cross cv. Californium and DH W-15 were studied concerning the number of branches per plant, the number of pods per plant, the number of seeds per pod, and the content of unsaturated C18 acids and glucosinolates. From among 210 studied DH lines, 26 DH lines exhibited positive transgression effects. The highest number of transgressive lines was recorded for the number of pods per plant (Szała et al., 2009a).

A total of 178 DH lines obtained from F1 hybrids of the reciprocal crosses between yellow-seeded DH Z-114 and black-seeded DH H_2-26 were studied with regard to the occurrence of transgression effects – positive for protein, oleic acid, linoleic acid, and fat content and negative for content acid detergent fiber, neutral detergent fiber, linolenic acid, and glucosinolate content. The highest number of transgressive segregants was observed for oleic acid in oil and protein content (Szała et al., 2011).

The general and specific combining ability

DHs have been successfully used for commercial production of new cultivars of oilseed rape. The usefulness of DH lines in the breeding of new varieties depends on their combined values. A multidimensional approach was adopted for the investigation of the general and specific combining ability of DH lines (parental forms) of winter oilseed rape from experiments with F1 hybrids obtained from the line × tester mating design. The general combining ability (GCA) and specific combining ability (SCA) effects of seven different DH lines and four testers were estimated for the yield components and the fat content (Adamska et al., 2007) and for five saturated and unsaturated fatty acids such as palmitic, stearic, oleic, linoleic and linolenic acids (Adamska et al., 2008). Selected testers included a high-yielding winter oilseed rape (cv. Lisek), a DH line with short pods, a yellow-seeded DH line and a DH line with high oil content. A statistical analysis of DH lines made it possible to distinguish two lines with especially advantageous values for the number of seeds per pod, the number of branches and the number of pods per plant. In addition, three pairs of lines and testers with significant multidimensional SCA effects and at least three positive and significant effects of individual yield structure traits were identified. Two lines showed positive GCA effects for oleic acid content and one of them showed, simultaneously, significant and negative GCA effects for the linolenic acid content.

Another attempt was made to examine the relationship between the genetic distance of parental forms and their phenotypic diversity. This was assessed by the line × tester analysis (Szała et al., 2009b), while the genetic diversity of parental forms was estimated via molecular Random Amplified Polymorphic DNA (RAPD) markers. The assessment of the phenotypic differentiation of

these forms was used to calculate the effects of heterosis, the GCA and SCA of the lines and testers and the Mahalanobis distances (Mahalanobis, 1936) between them.

There was a significant positive correlation between the genetic distance between the DH lines and the differences in the effects of GCA on the number of branches per plant. The relationship between the genetic distance between the lines and Mahalanobis distances proved to be significant for all genotypes (lines and testers) as well as for their corresponding Mahalanobis distances assigned to the six traits studied together.

Genetic variation in DH populations

DH lines are important source of a new genetic variation in the breeding of winter oilseed rape, which is a classic example of a species with a very narrow gene pool. If F1 hybrids are valuable, it is possible to count the desired segregation and recombination in gamete cells, and thereby obtain DH lines with agriculturally useful characteristics. Their adequate numbers can provide the greatest possible variability within the population resulting from a single hybrid. A significant variability can be obtained even in the case of haploidization of a cultivar, as evidenced by work on a 100 DH lines derived from a winter oilseed rape cv. Bor (Szała et al., 2002). DH lines differed significantly in terms of 9 of the 11 studied traits (excluding plant height and the timing of the onset of flowering).

However, the highest variation in a population of the DH lines can be obtained from hybrids whose parental components are phenotypically diverse and which have different origins. This was the case for a mapping population of more than 300 DH lines produced from an F1 hybrid resulting from crosses of high erucic acid and low glucosinolate content DH line ER-13/1 and zero erucic acid and high glucosinolate content DH line JN-86. As was revealed in a statistical analysis conducted for four morphological traits, the DH population developed to identify the quantitative trait loci (QTL) controlling the erucic acid and glucosinolate content was also characterized by a relatively high phenotypic variability (Szała et al., 2003).

The aim of the next study was to estimate the diversity of DH populations in terms of the yield, the yield structure, fat content and the levels of three fatty acids: oleic, linoleic and linolenic (Szała et al., 2013). This study was undertaken to determine if there was a correlation between the studied traits and their heritability, and to make clusters of the studied objects in terms of several traits together. Two DH populations were derived from F1 hybrids resulting from reciprocal crosses between cv. Californium and DH line W-15. A statistical analysis of both populations, based on two years of experiments, revealed that the greatest variability was characteristic of the number of pods per plant, while the lowest characterized the fat and oleic acid content. The seed yield was positively correlated with the number of branches and the number of pods per plant, while negatively correlated with a thousand seed weight.

Yellow-seeded oilseed rape is a very important source of protein-rich meal for livestock feed. However, the introduction of a yellow seediness trait to oilseed rape results in a reduction in yield with lower agronomic performance. Therefore, it is necessary to improve yellow-seeded breeding materials by further crossing with high yielding black-seeded forms. Hence, the main goal of the studies, conducted for two DH populations derived from F1 hybrids resulting from reciprocal crosses between black- and yellow-seeded DH lines, was to estimate the diversity of DH lines as well as the selection of the best yellow-seeded genotypes. The study indicated the presence of a substantial genetic variability among the DH lines obtained from F1 hybrids of crosses between black-seeded and yellow-seeded DH lines. The greatest variability was recorded for seed color and yield (Szała, 2012).

Effects of the cross direction

The determination of the influence of the cytoplasmic and nuclear genetic maternal effects or the embryo effects for given traits allows to select the appropriate direction of crossing. The reciprocal DH populations are great material to examine the influence of the direction of the crossing on the expression of the studied traits. A method of contrasts can be used to compare the average values for traits between these populations.

From a two-year field experiment, an estimation was made on the effect of the crossing on yield, its components, and some biochemical characteristics (Szała et al., 2015). The experimental material consisted of two DH populations obtained from F1 hybrids of reciprocal cros-

Table 1. DH line of winter oilseed rape as a material for genetic and statistical studies – summary

Material		Objective of research	Reference
Origin	No. of DH lines		
F1 hybrid DH C-1041 × DH O-120	32	genetic parameters for yield components	Adamska et al. (2002)
		genetic parameters for fatty acids	Cegielska-Taras et al. (2005)
		genotype-environment interaction for yield component	Cegielska-Taras et al. (2007)
		genotype-environment interaction for fatty acids composition	Kaczmarek et al. (2008)
		multivariate evaluation	Adamska et al. (2004) Kaczmarek et al. (2005)
F1 hybrids cv. Californium × DH W-15 DH W-15 × cv. Californium	210	transgression effects	Kaczmarek et al. (2009) Szała et al. (2009)
	38	genetic variability, multidimensional analysis – hierarchical clustering	Szała et al. (2013)
		cross direction effects	Szała et al. (2015)
		genotype-environment interaction	Szała (2012)
F1 hybrids DH Z-114 × DH H_2-26 DH H_2-26 × DH Z-114	176	transgression effects	Szała et al. (2011)
	44	genetic variability	Szała (2012)
		cross direction effects	Szała (2012)
Different	7 DH as a lines 3DH as a testers	multidimensional GCA and SCA effects for yield components	Adamska et al. (2007)
		GCA and SCA effects for fatty acids content	Adamska et al. (2008)
		genetic distance and GCA and SCA effects	Szała et al. (2009)
		genotype-environment interaction for unsaturated fatty acid content	Kaczmarek et al. (2011)
		multidimensional GCA and SCA effects for yield components	Adamska and al. (2007)
cv. Bor	100	genetic variability	Szała et al. (2002)
F1 hybrid DH ER-13/1 × JN-86	300	genetic variability	Szała et al. (2003)
F3 hybrid 3859/86	8	genotype-environment interaction	Adamska et al. (2000)

ses between cv. Californium and DH W-15 line derived from cv. Wotan, and the parental forms. In order to conduct such estimation, notice had to be taken of the significant variation between parental forms in the trait and the fact that the basis for inferences about the influence of maternal or paternal effects was the statistically significant differences between the mean values of the DH lines of the two populations. Both, the pair of parental forms and the pair of DH line populations revealed significant differences in the number of seeds per pod, and linolenic acid. Maternal effects have been revealed only for the number of seeds per pod.

A method of contrasts was used to compare the average values for seed color, yield components, and seed

quality between two populations of winter oilseed rape obtained from F1 hybrids of reciprocal crosses between the black-seeded DH line H_2-26 and the yellow-seeded DH line Z-114, and the parental forms. DH line Z-114 was obtained from a cross segregating for seed color between a natural mutant with bright seeds and a spring line of *B. napus*. The maternal effects were revealed in DH line populations only for the thousand seed weight. In contrast, the influence of the paternal form was found in terms of the content of neutral and acid fiber and seed color (Szała, 2012).

Genotype-environment interactions

In the breeding of new varieties, the selection for crossbreeding of appropriate parental components with high hereditary agricultural stability plays an important role. The desired properties do not only depend on the genotype, but also on different genotype responses to the environmental factors. Hence, more and more importance is being attached to the assessment of the yield stability and adaptability of new genotypes in different environments. Stable lines, characterized by broad adaptation to different climate and soil conditions, are particularly appreciated. However, genotypes that are narrowly adapted to specific environmental conditions, but which nonetheless show a true yield, are also sought. The first detailed analysis of a genotype-environment (G × E) interaction in seed yield of DH lines of winter oilseed rape was carried out for 8 DH lines, derived from anther culture of F3 hybrid and cv. Mar as a control, and based on the data from a series of experiments conducted at five locations for two years (Adamska et al., 2000). The experimental results were analyzed using uni- and multi-dimensional statistical methods that allowed an evaluation of the DH lines tested in terms of the amount of yield, and considering different aspects of their interactions with the environment.

An analysis of the interactions of the various genotypes with the environment using linear regression made it possible to distinguish two high seed-yielding DH lines: stable (C-1168) and unstable (C-1074). A similar analysis of the interactions of 38 winter oilseed rape DH lines derived from F1 hybrids and their parental forms with environment as well as yield stability assessment were conducted from results obtained over three growing seasons. It was found that the four highest yielding DH lines showed no interaction with the environment, and, of the six lines sensitive to environmental changes, three lines were defined as intense (Szała et al., 2011).

The other experiments were conducted with 32 DHs of winter oilseed rape, their parental forms, F2 and F3 segregating generations and the standard cv. Kana in six environments. This procedure enabled to determine the environmental and G × E interaction effects on some yield components and the fatty acid content. A statistical analysis of the results made it possible to evaluate DH lines in terms of different aspects of their environmental interactions for the length of the pod, the number of seeds per pod, and the thousand seed weight. It was also possible to distinguish a group of the best DH lines with respect to all the studied traits and to differentiate the stable DH lines for these traits and DH lines which showed a significant interaction with the environment (Cegielska-Taras et al., 2007). Using uni- and multivariate statistical methods, DH lines were also estimated under various environmental conditions in terms of the content and stability of two different fatty acid compositions. Kaczmarek et al. (2008) recommended using a model of variance analysis and related statistical methods to evaluate and select genotypes for industrial purposes. A total of 14 genotypes were found acceptable (11 stable and 3 unstable) for the chemical industry and 10 genotypes were acceptable (6 stable and 4 unstable) for the biodiesel industry. One DH line proved to be particularly interesting as it had a very high and significant content of the sum of linoleic and linolenic fatty acid and, simultaneously, a significantly high main effect for seed yield. The line was stable and may be recommended for the use in the chemical industry. The other stable DH line revealed, simultaneously, higher contents of oleic and lower content of linolenic fatty acids than the mean of all studied genotypes with a very high seed yield. There was a significant difference in the levels between these two fatty acids because this DH line meets the requirements of the biodiesel industry.

Likewise, 28 F2 hybrids obtained by crossing seven lines and four testers in the line × tester mating design, formed the experimental material in three-year series of experiments. The goal of these experiments was the evaluation of the stability of combining ability for unsaturated fatty acid content. Statistical methods made it possible to find two lines and one tester where GCA proved

to be high in the case of oleic acid and low in the case of linolenic acid (HOLL), i.e. having properties that are advantageous for biodiesel production. It should be added that the general combining ability of one of these lines was insensitive to the environmental conditions, whereas high significant interactions between genotype and year were found for GCA effect for the oleic acid content of a second line and tester (Kaczmarek et al., 2011).

Multidimensional analysis

The breeding programs of many plant species have aimed at improving of a number of traits that comprise the aspect of productivity and the quality of the crop. The starting materials are thus evaluated for many traits at the early stages of the plant breeding process. Multivariate statistical methods provide a comprehensive assessment of breeding lines while considering many important agronomic characteristics. They also allow for the separation of groups of similar genotypes in respect to many traits as well as the identification of groups of varieties of high value.

In a study of oilseed rape, multivariate statistical methods have been applied by Adamska et al. (2004). They identified genotypes with improved levels of oleic acid and characterized by the ratio of linoleic acid to linolenic acid 2:1 and the saturated fatty acid content of less than 7%, thus fulfilling the three basic criteria for wholesome edible oil. Kaczmarek et al. (2005) evaluated the DH lines in terms of the content of five fatty acids, further Adamska et al. (2007) evaluated the combining ability of DH lines in terms of the yield components and fat content.

In a multivariate analysis, the Mahalanobis distance is used as a measure of similarity between two objects. Based on the Mahalanobis distance, the hierarchical clustering was performed using the Ward method (Ward 1963) to classify the studied DH lines in terms of several traits jointly. A multidimensional analysis of DH lines and their parental forms showed their high level of diversity but it was not possible to establish a group of genotypes with the best agronomic performance (Szała et al., 2013).

This review of earlier studies has confirmed numerous possibilities of using winter oilseed rape DH lines in a statistical study. Owing to their genetic stability, DH lines can be tested in a series of experiments over many years, which allows for efficient selection of desired genotypes and their examination for adaptation in various environments.

Acknowledgements

This work was partially supported by the Ministry of Agriculture and Rural Development, Poland Project HORhn No 47.

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BioTechnologia vol. 96(2) • pp. 179-183 • 2015
Journal of Biotechnology, Computational Biology and Bionanotechnology RESEARCH PAPER

Effect of 2,4-dichlorophenoxyacetic acid pretreatment of *Capsicum* spp. donor plants on the anther culture efficiency of lines selected by capsaicinoid content

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Abstract

Androgenic low-responsive and non-responsive lines obtained as a result of the selection within the hybrid population of *Capsicum frutescens* L. × *C. annuum* L. were used for the determination of the pretreatment effect of donor plant with 2,4-dichlorophenoxyacetic acid (2,4-D) on the anther culture effectiveness. A significant increase in embryogenesis of the explants derived from pretreated plants showed an advantageous effect of 2,4-D application. The number of plantlets obtained was 1 to 18 for control and treated plant, respectively. About one half of the plants produced in the anther culture were diploids, characterized by 2C nuclear DNA content (1C/2C – 9/10). Callus tissues were mixoploid and comprised cells from 1C to 8C or from 2C to 16C of the nuclear DNA content. The microspores were the source of the first group of the callus tissue mentioned above. Spontaneous diploidization could be the reason for diploid plantlets regeneration from microspores. Obtaining regenerants in anther culture of a non-responsive hot line, in the case when donor plants were treated with 2,4-D, is the most interesting result of the experiment.

Key words: DNA content, haploid, diploid, callus, microspores

Introduction

The induced androgenesis allows for the rapid production of genetically stable recombinants being the original initial plant material for the breeding program of the innovative *Capsicum* spp. cultivars. Unfortunately, the pungent genotypes, the source of capsaicinoids, are recalcitrant in the androgenesis. The results of the investigation of the *in vitro* androgenesis of *Capsicum* spp. show genotype properties as particularly important for the effectiveness of the process. Sweet-fruited cultivars are a better source of the microspore-derived haploids. However, a great variation across genotypes occurs among these cultivars (Mityko et al., 1995; Regner, 1996; Rodeva et al., 2004; Dunwell 2010; Irikova et al., 2011). Genotype-dependent differences in organogenesis and somatic embryogenesis of pepper are also improved (Dabauza and Peña, 2001; Kaparakis and Alderson, 2008). In addition, the embryogenesis efficacy is highly influenced by the age of the donor plants (Ercan et al., 2006). Agronomic importance of *C. annuum* L. made this species the objective of experiments on induced androgenic embryogenesis in the *Capsicum* genus. The effective crossing between *C. frutescens* L. and *C. annuum* L. (Silva Monteiro et al., 2011) enables interspecific hybridization to be the source of a new genetic variation explored by androgenesis.

In the effective procedures of culture, a special role is played by 2,4-D as an androgenesis-inducing factor (Dumas de Vaulx et al., 1981; Supena et al., 2006). In the currently published modifications of the androgenic procedures, a special attention is paid to the improvement of media, their composition, and the treatment of explants (Koleva-Gudeva et al., 2007; Lantos et al., 2009). This growth regulator increases the fluency of polyembryony and haploid embryo frequency when plants are treated during florescence (Jędrzejczyk and Nowaczyk, 2009).

The crossing with highly effective forms is suggested to enhance the efficiency of androgenesis of cultivars without androgenic response. Unfortunately, creating new forms in this way causes undesirable changes in the plant genotype. In search of simple and low-cost androgenesis induction methods for non-responsive *Capsicum* spp., attention has been turned to plant material treatment before *in vitro* culture initiation.

The aim of the study was to evaluate the effect of the donor plant pretreatment with 2,4-D before the collection of flower buds, on the androgenic embryogenesis effectiveness in the *in vitro* anther culture of low-responsive and non-responsive lines selected from *C. frutescens* L. and *C. annuum* L. hybrids.

Material and methods

Besides agronomical values, for better characterization of the used plant material in this experiment, an analysis of capsaicinoid content was performed. Certain morphological and physiological properties of 30 fruits of each of the examined accessions were evaluated because of the plant material origin. As described by Collins et al. (1995), HPLC technique was applied for the analysis of capsaicinoid content in the whole fruit. Ground material samples (1.5 g) were separated into 50 ml glass tubes and submerged in 15 ml of acetonitrile. A Perkin Elmer Series 200 HPLC instrument equipped with Waters S5ODS2 4.6 × 100 mm column, autosampler, and PE Nelson Network Chromatography Interface NC 1900 was used. Standards of 8-methyl-N-vanillyl-6-nonenaminde (capsaicin) and 8-methyl-N-vanillyl-nonenaminde (dihydrocapsaicin) were provided by Sigma-Aldrich Co.

The anthers of 3 F7 lines, denoted 335, 342, and 345, derived through individual selection with progeny evaluation among interspecific *Capsicum frutescens* L. (female parent) and *C. annuum* L. hybrids, characterized by soft-flesh pericarp and capsaicinoid content, constituted the research material. Donor plants were grown in a plastic tunnel. Of about 20 individuals of each genotype were treated with 0.1 mg/l 2,4-dichlorophenoxyacetic acid water solution by spraying the whole plant about 12 h prior to bud collection. The buds' calyx and corolla were used and were similar in length, and most of the microspores were at the late-uninucleate stage.

Anther cultures of *C. annuum* L. were conducted in the way as described elsewhere (Dumas de Vaulx et al., 1981). The flower buds were surface-sterilized first by spraying with 70% ethanol and later by shaking in 5% solution of calcium hypochlorite (15 min). Next, they were rinsed thrice with sterile water. Anthers without filaments, isolated from two buds (on average 12 anthers), were placed on a Petri dish, with their inner parts facing the medium. For each genotype and treatment, an average of 75 Petri dishes (900 ± 10 anthers) was used. The anthers were cultured on a CP induction medium containing 0.01 mg/l 2,4-D and 0.01 mg/l KIN (kinetin). For the first 8 days, anther cultures were incubated in the darkness at 35 °C. Then, the dishes were exposed to a 12-h photoperiod at 25 °C. After 14 days, the anthers were transferred onto a R_1 regeneration medium (containing 0.1 mg/l KIN). In all the experiments, Gelrite (3 g/l) was used to solidify the media. The embryos that occurred in anther cultures were transferred onto V3 (Dumas de Vaulx et al., 1981) medium without growth regulators. Well-developed plants were then planted into the peat substrate and acclimatized in a plastic tunnel at increased air humidity.

The ploidy of plants, derived from anther culture and from callus, was assessed by the analysis of nuclear DNA content using flow cytometry. Samples were prepared following the Galbraith et al. (1983) procedure. The samples were analyzed with Partec CCA (Partec GmbH, Münster, Germany) flow cytometer equipped with a mercury lamp (High Pressure Lamp HBO-100W). The external standard used for cytometer calibration was a diploid plant of annual pepper *C. annuum* L. ($2n = 2x = 24$).

The results of donor plant evaluation and the effectiveness of anther cultures were the subject of statistical analyses. The values of the Least Significant Difference were established using Tukey's test at $p = 0.05$.

Results and discussion

When planning the experiment, an important methodology problem was the determination of 2,4-D concentration and application period. Considering the protective effect of corolla petals, which make the growth regulator penetration difficult, a ten-time higher 2, 4-D concentration was used as adequate for the induction medium in the experiment. In addition, it was assumed that providing the donor plants with the growth regulator a dozen-or-so-hours in advance (when compared to the explant collection time) will be justifiable in terms of the length of microsporogenesis and the applicability of buds for anther culture. In other words, it was assumed that disturbing the hormonal balance at the beginning of that process *in vivo* can enhance the effectiveness of androgenesis in *in vitro* culture.

The pungent, soft-flesh, and genetically stable forms used in the experiment, produced as a result of the se-

Table 1. Characteristics of donor plant fruit of *Capsicum* spp. lines

Feature	Line		
	335	342	345
Mean weight [g]	38.0c	15.3b	8.5a
Length [mm]	115b	70a	63a
Width [mm]	40b	24a	18a
Wall thickness [mm]	2.63b	2.20a	3.00b
Soluble solids [°Brix]	8.0a	7.4a	8.1a
Dry matter [%]	12.1b	9.0a	8.6a
Capsaicinoid content [$mg \cdot g^{-1}$]			
Capsaicin	0.46b	0.09a	1.25c
Dihydrokapsaicin	0.13a	0.08a	0.57b
Technological performance [%]	71c	63b	46a

Data denoted by the same letter for feature are not significantly different

Table 2. Effectiveness of callus and embryo formation in anther *in vitro* culture of *Capsicum* spp. lines according to 2,4-D donor plant treatment

Line and treatment	Callus		Embryos	Plants	Number of 1C/2Cplants
	Number per 100 anthers	DNA content	Number per 100 anthers		
335	3.66bc	2C – 16C	0.22a	0.22a	1/0
335 (2.4D)	4.67cd	1C – 8C	0.89b	0.67b	3/3
342	0.03a	1C – 8C	0.0a	0.0a	0
342 (2.4D)	1.22ab	2C – 16C	0.67b	0.67b	1/5
345	4.11c	2C – 16C	0.22a	0.0a	0
345 (2.4D)	6.67d	2C – 16C	0.67b	0.67b	4/2

Data denoted by the same letter for feature are not significantly different

lection within interspecific hybrid population, differed in their morphology and physiology (Table 1). As for the technological characteristics, special attention was paid to the capsaicinoid content, as it is common knowledge that pungent pepper genotypes are very low androgenic-responsive. The created genotypes are initial material suitable for the next breeding step, and from this perspective, their androgenic response should be further evaluated.

The effectiveness of anther culture was low for the studied genotypes, mostly due to the genetic properties of plant material (Table 2). In total, the number of 19 acclimatized regenerants obtained by us, and 15 derived by Lantos et al. (2009) from microspore culture of 6 hot pepper cultivars, seems to be similar. Depending on the cultivar, the mean number of plants per Petri dish in the experiment ranged from 0% to 1.25%. Koleva-Gudeva et al. (2007) presented the results of the embryogenesis induction on the CP medium similar to one that was used in the present experiment and reported androgenic effectiveness ranging from 0% to 55% (measured as a ratio between the number of embryos and the number of anthers). There were no embryos produced when a pungent donor plant was used. Similar results were recorded by Mityko and Fari (1997) in the experiment with "Serrano" chili pepper. The same physiological pepper type was also included in the present research. Investigating individual reactions of F2 plants of inter- and intra-specific hybrids within the *Capsicum* genus emphasize the effect of the particular genotype on andro-

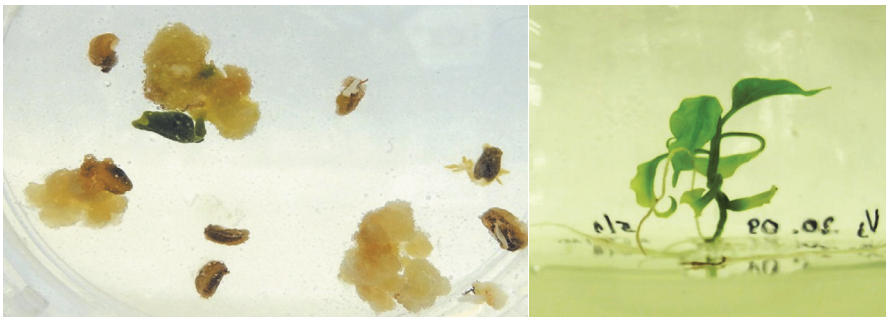

Fig. 1. Callus papules on anthers (R_1 medium) and plantlet (V3 medium) of *Capsicum* spp.

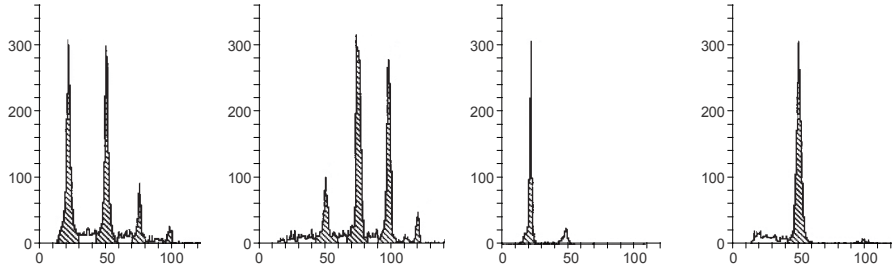

Fig. 2. Histograms of *Capsicum* spp. DNA content in callus cells of 335 line: 1C – 8C (first), 2C – 16C (second); in plantlets cells of 345 line: 1C (third), 2C (fourth)

genesis induction (Nowaczyk et al., 2009). Among other factors that differentiate the androgenic response, the type of explant appears to be important. The high androgenic response can be obtained by shed-microspore cultures. This was confirmed in the studies by Supena et al. (2006) and Kim et al. (2008).

For each of the genotypes studied here, it was noted that explants from the treated donor plants demonstrated a higher androgenic response than untreated ones, suggesting an advantageous impact of 2,4-D. This applies to both callus tissue and androgenic structures. As an effect of the pretreatment of anthers *in vivo*, the number of embryos in anther culture *in vitro* increased. However, the results should be treated with caution before those tendencies are confirmed using a greater number of genotypes and applying different plant treatments.

The conversion of an embryo to a plant is the crucial point of embryogenesis. In the experiment described by Supena et al. (2006), an effective conversion was only observed in some of the embryos. In their latest work on refinement of shed-microspore culture protocol for hot *Capsicum annuum* L., Supena and Custers (2011) reported a high percentage of "regular-looking" embryos. Unfortunately, in the experiment, only one genotype was used, and there is no information about the effectiveness of the embryo conversion. A considerable conversion success in the present experiments seems promising, however, limited by the number of accessions under investigation (Table 2, Fig. 1). The flow cytometry analysis showed the presence of nuclei with 1C or 2C DNA (Fig. 2). The number of diploids was surprisingly high and made the classification of the embryogenesis type difficult. The origin of the somatic tissue of diploid plantlets cannot be excluded. However, on the contrary, a spontaneous diploidization of androgenic embryo is also possible. A polyploidization was noted in the callus tissue. Histograms show a high diversification of 335 line DNA content. Similarly, a spontaneous diploidization was observed by Lantos et al. (2009) in microspore cultures. There were regenerated 15 diploid plants, and only three of them were produced as a result of the application of colchicine. The others, accounting for 80% of the population of regenerates, were spontaneous diploids of androgenic origin. Parra-Vega et al. (2013) confirmed a gametophytic origin of all tested embryos derived from *Capsicum annuum* L. anther culture.

The histogram (Fig. 2) demonstrating the presence of nuclei with 1C-8C DNA content in callus suggests a microspore origin of the tissue. A spontaneous polyploidization is the result of the creation of the cells with

a higher DNA content. A similar situation was typical for callus developed from the somatic cells of the anther wall, and in this case, the DNA content ranged from 2C to 16C. The chromosome number doubling in *Capsicum annuum* L. microspores *in vitro* culture described by Lantos et al. (2009) confirms the androgenic origin of diploids. Evaluating the results, one shall consider the simplicity and low cost of the explant pretreatment. The effects call for further experiments on 2,4-D concentration and the time of plant pretreatment.

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BioTechnologia vol. 96(2) • pp. 184-191 • 2015
Journal of Biotechnology, Computational Biology and Bionanotechnology RESEARCH PAPER

Interspecific hybridization between *Brassica napus* and *Brassica rapa* ssp. *chinensis* genotypes through embryo rescue and their evaluation for crossability

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Abstract

The genus *Brassica* contains several important crop species that are used for a variety of purposes. *Brassica napus* and *Brassica rapa* are the most important ones for their use as oilseed crops. In *Brassica*, interspecific hybridization is a potential and useful method for transferring valuable traits between species of commercial interest. For breeding Brassicas resistant to some diseases, i.e. clubroot, attempts of transferring resistant genes (CR) through interspecific hybridization have also been reported. The main step in the introgression process is the production of interspecific hybrids between the two species. However, the cross-incompatibility occurring in wide hybridization might hamper the possibility of obtaining hybrid progenies. For that reason, the crossability study is essential and may give an insight into the cross-compatibility relationship among the species, the direction of success of crossing, and the crossability barriers of some combinations, if any. In the present study, interspecific reciprocal crosses between *B. napus* and *B. rapa* ssp. *chinensis* genotypes were carried out in order to determine their crossability and to produce their F1 hybrids. Crossability was analyzed based on the pollen germination index (PGI) and the development of hybrid embryos in *in vitro* cultures. It was observed that the unilateral interspecific incompatibility occurred in crosses between *B. napus* × *B. rapa* ssp. *chinensis.* When *B. napus* cultivars were used as a maternal parent, pollen grains of *B. rapa* germinated well on the stigmas, while only a small number of pollen tubes could elongate near the ovules, resulting in a low development of hybrid embryos. Using the *B. rapa* ssp. *chinensis* as the pistillate parent in the crosses, it was found that the crossability was relatively higher than that in the case of the reciprocal cross. This resulted in a greater number of embryos obtained. Generally, it was concluded that the selection of parental components for hybridization is an important step for obtaining breeding success.

Key words: interspecific hybridization, crossability, embryo rescue, Brassicas, clubroot resistance

Introduction

Many *Brassica* species and allies are wild and weedy with useful genes which could be incorporated into breeding programs, including research into the cytoplasmic and nuclear male sterility; the resistance to diseases; insect or nematode pests; and the tolerance of cold, salt, and drought conditions (Chen et al., 2011). Thus, interspecific hybridization is still a useful approach for the introgression of these favorable agronomic traits from wild species into cultivated crops (Mei et al., 2010; Niemann et al., 2012, 2014). It is interesting, mainly because of the growing yield losses in oilseed rape caused by various pathogens among which one of the most dangerous is *Plasmodiophora brassicae* (Pageau et al., 2006). For breeding Brassicas resistant to clubroot, attempts of transferring CR through interspecific hybridization have been reported (Gowers, 1982; Diederichsen et al., 2009; Niemann et al., 2015). Among the two progenitor species of *Brassica napus*, clubroot resistance is more frequently found in turnips (*Brassica rapa*; A genome; $n = 10$) (Hirai, 2006). This is why the crosses between *B. napus* and the three genotypes of *B. rapa* ssp. *chinensis* with potentially increased resistance to clubroot were made. However, interspecific hybridization between allotetraploid and diploid *Brassica* species is difficult; as a result, cross-incompatibility often hampers the production of those hybrid progenies. While most attempts on interspecific hybridization were based on conventional techniques (hand pollination), the frequency of hybrids was low. In addition, hybrids were obtained at relatively higher

frequency when *B. rapa* was used as a female parent, indicating a strong incompatibility in the reverse cross (Ammitzboll et al., 2005). Therefore, the main goal of our study was to evaluate the crossability between *B. napus* and *B. rapa* ssp. *chinensis* genotypes and to obtain F1 hybrids between those genotypes using an embryo rescue technique.

Materials and methods

Plant materials and crosses

Five *B. napus* seed cultivars, i.e. Jet Neuf, Lisek, Skrzeszowicki, Californium, and Zhongshuang 9, were selected from the resources of the Genetics and Plant Breeding Department, Poznań University of Life Sciences, while seeds of *B. rapa* accessions (A: Chinense Cabbage PI430485 98CI; B: Pak Choi 08 007569; C: Chinense Cabbage 08 006169) came from the Warwick HRI Genetic Resources Unit. The reciprocal crosses were carried out in the glasshouse of Genetics and Plant Breeding Department during spring 2013. Each genotype was represented by four to six plants. The pollen of pollinators was placed on stigmas immediately after emasculation, which was done at the closed bud stage. In each cross-combination, around 11 to 90 flower buds were pollinated.

Pollinated pistils were divided into two parts. Half of them were allocated for the microscopic examinations. The rest of pistils were left on the plants to silique formation.

The evaluation of crossability based on the PGI

Observations of pollen grain germination and pollen tube (PT) growth were conducted after cross-pollination (CP) of the chosen *Brassica* genotypes. For this purpose, pistils of three *B. napus* cultivars i.e. Jet Neuf, Californium, Skrzeszowicki, and one *B. rapa* ssp. *chinensis* genotype (08 006169) were collected 48 h after pollination. They were next fixed and stained with aniline blue (Antkowiak and Wojciechowski, 2006). By applying a fluorescent microscopy technique, the assessment of pollen tube growth was made. Six pistils were analyzed in each combination. The intensity of pollen grain germination and pollen tube penetration was expressed by the six degree scale (Niemann et al., 2014), where 0 was the absence of PT, 1-4 was the intermediate number of PT, and 5 was the largest number of PT. The crossability (CC: cross-compatibility; CI: cross-incompatibility) was evaluated on the basis of the PGI according to Kaneko et al. (2009); and also, pistils were classified into five specimens: 1) the number of pistils with pollen grains, 2) the number of pistils in which pollen grains do not germinate, 3) the number of pistils in which pollen grains germinate on the stigmas, 4) the number of pistils in which pollen tubes enter the style tissue, and 5) the number of pistils in which pollen tubes penetrate close to or enter the ovules.

The value of PGI was obtained from the formula of PGI = (1b+2c+3d+4e)/(a+b+c+d+e).

In the case of PGI equal or higher than 2, it was concluded that there existed compatibility.

The effectiveness of crosses

All hybridizations were performed with the application of an *in vitro* culture of isolated embryos according to the method described by Wojciechowski (1998).

The immature embryos were isolated from young siliques at different developmental stages, i.e. heart, early torpedo, torpedo, late torpedo, or nearly matured embryos, 14-19 days after pollination. For the embryo culture, basal White (W, 1963), Murashige and Skoog (MS, 1962), Murashige and Skoog modified by Keller and Armstrong (Ms_k, 1977), and Nitsh and Nitsh (H_3, 1969) media were applied (Table 1).

The effectiveness of an interspecific hybridization of *B. napus* with chosen *B. rapa* genotypes was expressed in three different ways: by the number of embryos obtained to the mean number of well-developed ovules, by the fertility (siliqua/pollinated flowers (%) – Figure 1), and by the number of plants regenerated in the soil.

Results

Evaluation of crossability based on the PGI

Generally, a different intensity of pollen tube growth and the differences at PGI value were observed in most cross-combinations, depending on which form was used as a maternal parent. It was observed that after cross-pollination of *B. napus* cultivars with *B. rapa* ssp. *chinensis* pollen grains, the intensity of pollen tube growth was low, and the pollen tubes were observed mainly in the style or in the ovary. Occasionally, a low number of ovules were penetrated by pollen tubes (PGI value less than 2) – Figure 2E. The crossing between three *B. na-*

Table 1. Pattern of *Brassica* embryos cultures in *in vitro* conditions

No.	Media	Embryo/plant developmental stage	*In vitro* culture duration	Conditions of *in vitro* cultures
1.	White	H*, ET	7 days	Growth room**
2.	Murashige & Skoog	T, LT, NM	3-4 weeks	
3.	Murashige & Skoog in Keller modification	explants developing callus and first leaves	5-11 weeks	
4.	Nitsch & Nitsch	seedlings	2 weeks	
5.	Soil	well rooted seedlings	7 days	
			until matured plants	greenhouse

* H – heart, ET – early torpedo stage, T – torpedo stage, LT – late torpedo, NM – nearly matured embryo,
** conditions: temperature 26°C; photoperiod: 16 h light, 8 h dark

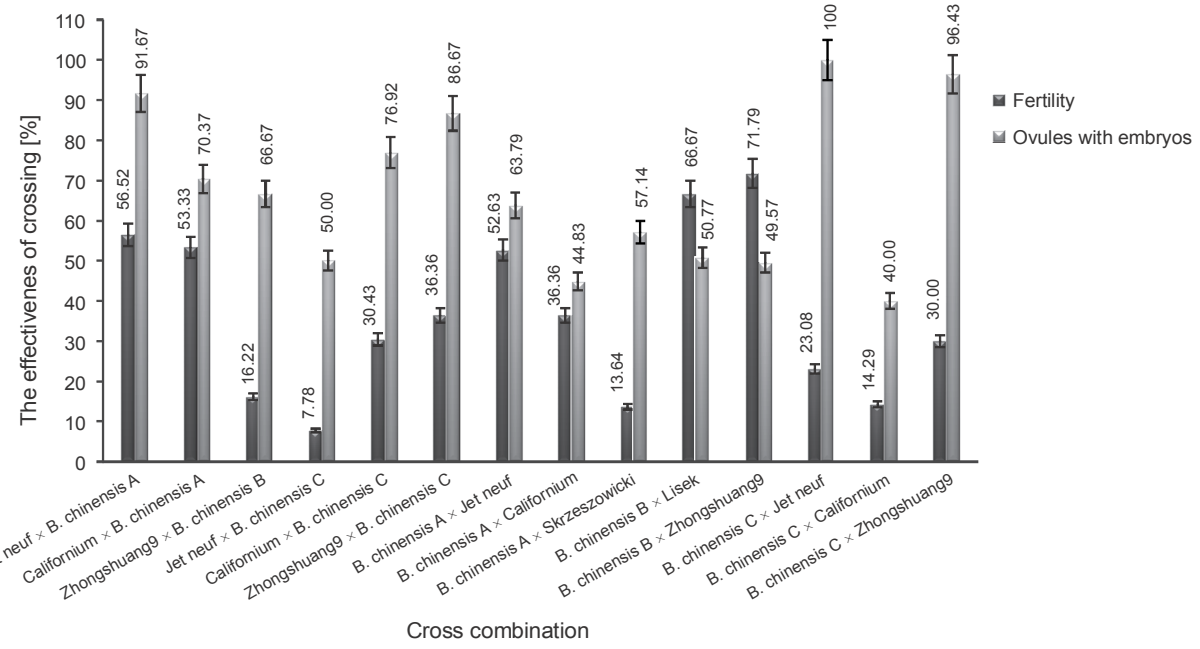

Fig. 1. Effectiveness of reciprocal crosses between *B. napus* cultivars with *B. rapa* ssp. *chinensis* genotypes expressed by the fertility and ovules with embryos (%). Error bars indicate within subject standard error

pus cultivars and the tested genotype of *B. rapa* ssp. *chinensis* showed that these two species do not suit each other concerning their compatibility, and in these cases, there are some prezygotic incompatibility barriers. Quite a different situation was observed in crosses in which *B. rapa* ssp. *chinensis* plants were used as a maternal and *B. napus* cultivars as a pollen donor. In those cases, the intensity of pollen tube growth was higher, and PGI ranged from 2.11 (*B. rapa* ssp. *chinensis* × *B. napus* cv. Californium) to 2.69 (*B. rapa* ssp. *Chinensis* × *B. napus* cv. Jet Neuf) with a mean value of 2.32 (Table 2, Fig. 2A-D).

The effectiveness of crosses

The highest fertility was observed in crosses between *B. rapa* ssp. *chinensis* B and *B. napus* cv. Lisek and Zhongshuang 9. It was 66.67% and 71.79%, respectively (Fig. 1). Whereas in one of the reverse crossing, i.e. *B. napus* cv. Lisek × *B. rapa* ssp. *chinensis* B, there were no siliques obtained at all. Similarly, no siliques were received in *B. napus* cv. Lisek × *B. rapa* ssp. *chinensis* C and *B. napus* cv. Skrzeszowicki × *B. rapa* ssp. *chinensis* A. Moreover, in all cross-combinations, quite a high percentage of ovules with embryos were obtained

Table 2. Pollen germination index (PGI) in reciprocal crosses of chosen *Brassica* genotypes

Combination of pollination		PGI 48 h after pollination
B. napus cv. Jet Neuf	*B. rapa* ssp. *chinensis* C	1.94
B. napus cv. Californium		1.81
B. napus cv. Skrzeszowicki		1.88
Mean		1.88 ± 0.06*
B. rapa ssp. c*hinensis* C	*B. napus* cv. Jet Neuf	2.69
	B. napus cv. Californium	2.11
	B. napus cv. Skrzeszowicki	2.17
Mean		2.32 ± 0.32

* Mean ± SD

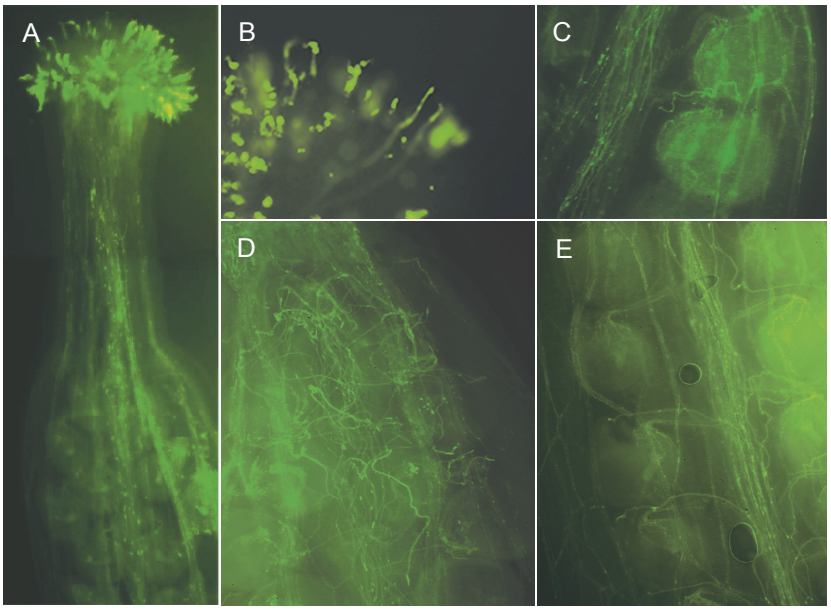

Fig. 2. Pollen germination and pollen tube elongation in pistils of analyzed *Brassica* genotypes: A) The pistil of *B. rapa* ssp. *chinensis* A after pollination with *B. napus* cv. Californium; B) Pollen grains germinating on the stigma, *B. napus* cv. Zhongshuang 9 × *B. rapa* ssp. *chinensis* A; C-D) pollen tubes in the ovary, *B. rapa* ssp. *chinensis* A × *B. napus* Jet Neuf; E) Pollen tube penetrating the ovule, *B. napus* cv. Lisek × *B. rapa* ssp. *chinensis* A

ranging from 40% in *B. rapa* ssp. *chinensis* C × *B. napus* cv. Californium to 96.43% in *B. rapa* ssp. *chinensis* C × *B. napus* cv. Zhongshuang 9. In the case of the last-mentioned cross-combination, plants were obtained from 51.85% of the isolated embryos.

In this experiment, from 236 pollinated flowers, up to 452 embryos (Fig. 3A) were received after crosses between all *B. rapa* ssp. *chinensis* × *B. napus* genotypes. In the case of reverse hybridization, a lower number of embryos were formed out only 80, despite the pollination of a greater number of flowers (273). However, the efficiency of embryo rescue presented as the ratio of the regenerated plants to the isolated embryos was higher in these variants of crosses, and it was 73.7% (*B. napus* × *B. rapa* ssp. *chinensis*), while only 35% of plants were regenerated from the *B. rapa* ssp. *chinensis* × *B. napus* crosses. Generally, in all tested cross-combinations, 509 flowers were pollinated and 220 plants were regenerated through embryo cultures, wherein most plants were obtained from the crosses between *B. rapa* ssp. *chinensis* × *B. napus* genotypes – 161 (Fig. 3B-F, Table 3). In those cross-combinations, the highest number of em-

Table 3. Effectiveness of interspecific crosses between *B. napus* cultivars and *B. rapa* ssp. *chinensis* genotypes expressed by the number of regenerated plants in the soil

Cross-combination				No. of pollinated flowers	No. of siliques	No. of isolated embryos				No. of plant rooted in H_3	No. of plant in soil
♀		♂				Total	In different developmental stages				
							H	ET	LT		
B. napus	Jet Neuf	*B. rapa ssp. chinensis* A		23	13	11	0	0	11	7	7
	Californium			15	8	19	0	8	11	12	12
	Skrzeszowicki			13	0	0	0	0	0	0	0
	Lisek	*B. rapa ssp. chinensis* B		11	0	0	0	0	0	0	0
	Zhongshuang9			37	6	2	0	0	2	0	0
	Jet Neuf	*B. rapa ssp. chinensis* C		90	7	2	0	0	2	2	2
	Lisek			28	0	0	0	0	0	0	0
	Californium			23	7	20	0	7	13	19	18
	Zhongshuang9			33	12	26	0	18	8	26	20
Total				273	53	80	0	33	47	66	59
B. rapa ssp. *chinensis* A		*B. napus*	Jet Neuf	19	10	74	24	50	0	22	22
			Californium	22	8	39	20	19	0	10	8
			Skrzeszowicki	66	9	68	12	50	6	60	57
B. rapa ssp. *chinensis* B			Lisek	15	10	66	29	35	2	0	0
			Zhongshuang9	39	28	173	64	80	29	61	59
B. rapa ssp. *chinensis* C			Jet Neuf	13	3	1	0	1	0	1	1
			Californium	42	6	4	1	3	0	0	0
			Zhongshuang9	20	6	27	0	0	27	14	14
Total				236	80	452	150	238	64	168	161
General				509	133	532	150	271	111	234	220

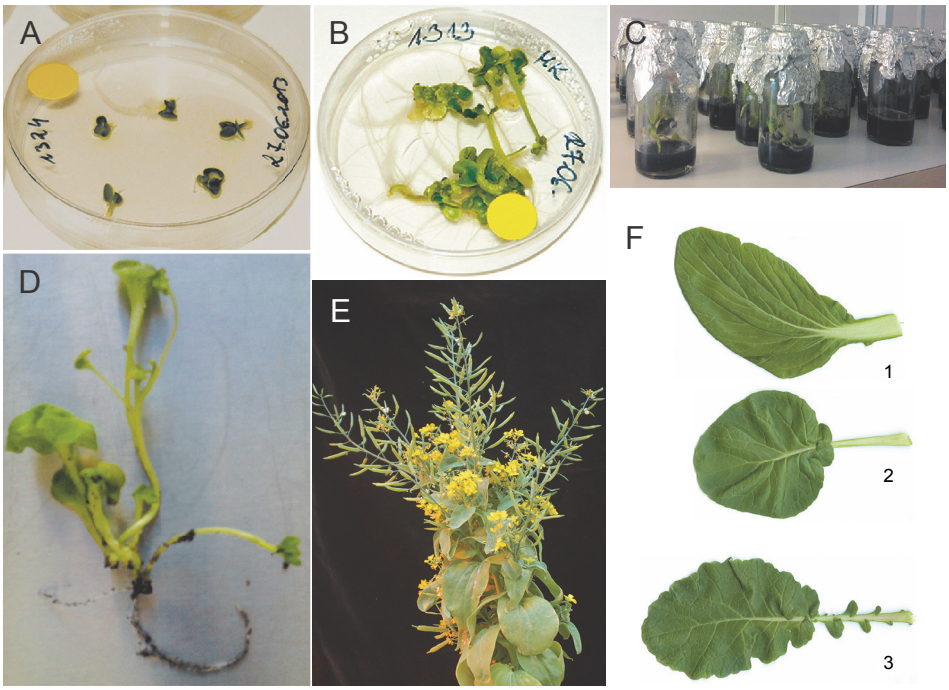

Fig. 3. Regeneration of plants in *in vitro* embryo cultures after crossing *B. rapa* ssp. *chinensis* A × *B. napus* cv. Jet Neuf: A) Fully matured embryos on MS medium; B) Regenerated seedlings on MS_k medium; C) Regenerated plant on rooting H_3 medium; D) Regenerated plant just before being transferred to soil; E) Regenerated plant in the soil; F) Comparative leaf morphology of F1 hybrid plants: 1 – *B. rapa*, 2 – F1 hybrids, 3 – *B. napus*

bryos was isolated at the heart and early torpedo stages, while after *B. napus* × *B. rapa* crosses, no embryos have been obtained at the heart developmental stage.

Discussion

To date, successful attempts of hybridization between *Brassica rapa* and *Brassica napus* have been published in numerous scientific papers (Fitz et al., 2007; Choudhary et al., 2012). Crossability between *B. napus* and *B. rapa* has also been examined, specifically in terms of the genes introgression from *B. napus* to *B. rapa*, which is mainly considered a weed in America and Europe (Tsuda et al., 2014). However, to the best of our knowledge, this is one of the first studies focusing directly on the potential of hybrid production and crossability between diploid *B. rapa* ssp. *chinensis* genotypes and allotetraploid *B. napus* in terms of the portability of the resistance genes. It is known that in *B. rapa*, a high level of genetic diversity was observed, and moreover, species of *B. rapa* vary in their level of cross-compatibility (Olsson, 1960).

Thus, it is difficult to compare our results with those presented in literature, particularly because *B. rapa* is a species consisting of various widely cultivated subspecies including *pekinensis*, *chinensis,* the turnip and the turnip rape (*B. rapa* subsp. *oleifera*). Within the species, different genotypes may differ in their propensity to cross with other species depending on their crossability or self-incompatibility. For this reason, experimental hybridization studies for plant breeding often incorporate diverse genotypes. This means that even if one genotype crosses with a wild relative, others within the same species may not. Although, according to Downey et al. (1980), *B. rapa* varieties are generally self-incompatible (Indian brown sarson and toria types as well as North American and European *B. rapa* cultivars), some of them are highly self-compatible (Indian yellow sarson form of *B. rapa*).

Despite the fact that *B. rapa* is one of the most comprehensively studied crops, as with *B. napus*, relatively few studies report success of hybrid production quantitatively (Fitz et al., 2007).

It is commonly known that canola (*B. napus,* AACC genome) and *B. rapa* (AA genome) have a common set of chromosomes, which facilitates interspecific gene flow between these two species. Spontaneous hybridization

in the field is possible, and natural *B. napus* × *B. rapa* hybrids have been reported in several countries, including Canada (Yoshimura et al., 2006), Denmark (Hansen et al., 2001), and the United Kingdom (Allainguillaume et al., 2006). In field trials, under natural conditions, the success of hybridization varies widely, depending on the experimental design and the direction of crosses (Pallett et al., 2006). However, subsequent introgression of alleles from *B. napus* to *B. rapa* occurs infrequently (Leflon et al., 2006). In the laboratory experiments, *B. rapa* pollen has a significantly lower degree of fitness on *B. napus* than conspecific pollen, and hybrid zygote survival is markedly reduced in comparison to conspecific zygotes (Hauser et al., 1997). In addition, *B. rapa* × *B. napus* hybrids have been found to have reduced fertility and lower seed set compared to either parental species (Jorgensen and Andersen, 1994).

The results of our investigation showed that the biggest number of embryos was received when *B. rapa* ssp. *chinensis* was treated as a female parent. These results are consisted with those obtained from the literature data (Ammitzbol et al., 2005) because they demonstrate that hybridization rates are significantly higher if *B. rapa* is a female parent, rather than vice versa, due to the self-incompatibility of *B. rapa*.

Our results confirmed that there is unilateral crossability between *B. rapa* ssp. *chinensis* and *B. napus* genotypes. When *B. rapa* ssp. *chinensis* was a female parent and *B. napus* was a male parent in all cases, cross-compatibility was observed with a mean PGI value = 2.32 ± 0.26. In reciprocal crosses, generally there was incompatibility (PGI = 1.87 ± 0.05*). According to Long et al. (1992), *pekinensis* group was more compatible with *Brassino raphanus* than *rapifera* group in *B. rapa.* In addition, kales were even more compatible than other varieties of *B. oleracea,* which suggests that the selection of certain species, subspecies, varieties or cultivars as pollen parents is important and may improve the cross-compatibility between them. Although, we obtained hybrid plants in both directions of crosses *B. rapa* ssp. *chinensis* × *B. napus* and *B. napus* × *B. rapa* ssp. *chinensis* (161 and 59, respectively). A greater number of regenerated plants were obtained when *B. rapa* ssp. *chinensis* was a female parent. Those results are coinciding with our observation of PGI value. This is particularly interesting in view of our previous studies on crossability in different *Brassica* species (Niemann et al., 2014). In those cases, crosses between self-compatible (SC) cultivar Californium (*B. napus*) and self-incompatible (SI) *B. rapa* ssp. *pekinensis* were successful only when SC cultivar was used as a maternal form. Reciprocal crosses (SI × SC) failed. Fitz et al. (2007) made a similar observation related to the crossability barriers. Crosses between *B. napus* and *B. rapa* (a progenitor of *B. napus*) were very successful with a median rate of 2.29 hybrids/pollination when *B. napus* was the female parent in the cross but lower (0.44 hybrids/pollination) when *B. napus* was the male parent in the cross. In both directions, the rates of production ranged widely across trials (Fitz et al., 2007). The same authors proved that crosses between *B. napus* and *B. rapa* were more successful when the amphidiploid species, i.e. *B. napus*, was used as a female parent. Reciprocal crosses were generally unsuccessful. Moreover, Choudhary et al. (2012) demonstrated that amongst crosses involving three varieties/forms of *B. rapa* with *B. napus*, yellow sarson produced a maximum number of hybrids (18.0%) followed by brown sarson (11.3%) and toria (6.9%). Thus, the success rate of cross-fertility of three varieties of *B. rapa* with *B. napus* was in the order yellow sarson > brown sarson > toria. These observations are similar to our results indicating that the success of interspecific crosses depends not only on the species but also on the form / varieties of the species involved. That is why we observed differences in the number of *B. napus* × *B. rapa* ssp. *chinensis* hybrids obtained through embryo rescue. The latest was probably related to interspecific crossability between genotypes.

Conclusions

1) Observations of the pollen tube growth and embryo set indicate unilateral interspecific compatibility in the crosses where *B. rapa* ssp. *chinensis* was used as a maternal form. However, the number of obtained embryos depended on the genotype.
2) The highest number of hybrids was obtained from crosses between *B. rapa* ssp. *chinensis* × *B. napus.*
3) The degree of hybridization between *B. rapa* and *B. napus* varied depending on subspecies of *B. rapa,* which were used in the crosses and the direction of crossing.

Acknowledgements

The Authors thanks to the Ministry of Agriculture and Rural Development, Poland for financial support of task no. 54.

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BioTechnologia vol. 96(2) • pp. 192-202 • 2015
Journal of Biotechnology, Computational Biology and Bionanotechnology RESEARCH PAPER

Determination of an optimal isolation and culture conditions of grass pea protoplasts

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Abstract

This paper presents a study on determination of optimal grass pea (*Lathyrus sativus* L.) protoplast isolation and culture conditions. The plant material comprised the Polish variety "Derek". Explants for protoplast isolation were leaves of 10, 15, 21 and 28 day-old *in vitro* and *in vivo* grown seedlings. The plant material was briefly incubated (3-4 hours) or left overnight (17-18 hours) in different enzymatic mixtures. The isolation efficiency and viability of protoplasts were assessed to compare the applied isolation conditions. The best selected isolation conditions were used in subsequent experiments. Protoplast cultures were established in liquid and solid media enriched in various supplements. Protoplast viability, morphological responses and cell wall reconstruction were evaluated. Grass pea leaves proved to be a good source of protoplasts. The origin and the age of donor plants as well as the type of the applied enzymatic mixture had an impact on the isolation efficiency, viability of protoplasts and further protoplast responses during the culture. Overnight incubation resulted in a higher yield of protoplasts. However, protoplasts isolated from briefly incubated material had higher viability. Protoplasts from leaves of 15-21 day-old *in vitro* seedlings obtained after overnight isolation showed the highest viability on the 10th day of cultivation. In liquid media, protoplasts survived for about 10 days and only an addition of chitosan prolonged their viability to more than 15 days. Shape changes and intensive budding of protoplasts were observed during the culture. Although no steady mitotic activity was observed in liquid media, occasional cell divisions were noted in an agarose-droplet culture. After 24 hours, grass pea mesophyll protoplasts rebuilt their cell wall at different ratios (10-60%) depending on the applied media. A high frequency of protoplast budding suggests some abnormalities in cell wall structure that prevent the further development of a culture.

Key words: agarose-droplet culture, chitosan, *Lathyrus sativus*, leaf mesophyll, protoplast isolation efficiency, protoplast viability

Introduction

The significance of grass pea (*Lathyrus sativus* L.), a grain-leguminous plant, in agriculture is constantly growing. The high protein content in the seeds, together with resistance to drought and tolerance to diseases and pests, make grass pea an outstanding, alternative source of protein. Additionally, grass pea plants grow and develop on poor soils and in harsh environmental conditions, in contrast to other high-protein legume crops (Campbell et al., 1994; Vaz Patto et al., 2006). However, despite the efforts of plant breeders, grass pea has a number of disadvantages, e.g. pod shattering, prostrate plant habit, late maturity, indeterminate growth, and anti-nutritional substances in seeds (Rybiński, 2003). All these features limit the spread of grass pea cultivation.

The improvement of the species is associated with the necessity of receiving new genotypes. Interspecific crossbreeding within the *Lathyrus* genus is difficult and, unfortunately, the reasons for this incompatibility are not known (Campbell, 1997). A problem is also the lack of some desirable traits in the available breeding materials. Consequently, conventional breeding based on recombination is not very effective. This forces the search for alternative methods of breeding. Besides mutation breeding, *in vitro* methods also enable the possible extension of the genetic variation of this species. Protoplast cultures enable new recombinants to be obtained by protoplast fusion and somatic hybridization. This is particularly important in the case of crossing barriers between parental components. Furthermore, the fusion

of grass pea protoplasts with pea protoplasts is desirable, because of the resistance of grass pea to *Ascochyta* blight (Campbell, 1997; Duriou and Ochatt, 2000). Owing to the absence of the cell wall, protoplasts are also excellent explants for genetic transformation. Notwithstanding this factor, efficient protoplast isolation and culture, as well as plant regeneration protocols, are indispensable to use all these techniques.

Although some protocols for plant regeneration from protoplast cultures have been developed for several grain legume crops (Ochatt et al., 2000; Dhir et al., 1992; Tegeder et al., 1995), there have been no reports on plant regeneration from protoplast cultures of the *Lathyrus* species. The first isolation of grass pea protoplasts was carried out by McCutchan from shoots and a cell suspension in 1999 (McCutchan, 2001). However, the obtained protoplasts did not exhibit the ability to divide. Durieu and Ochatt (2000) isolated protoplasts from embryonic axis shoots that subsequently divided and formed microcalli. Until now this has been the only report on dividing grass pea protoplasts.

The aim of this study was to develop optimal conditions for grass pea protoplast isolation and culture.

Materials and methods

Plant materials

The plant material was a Polish variety of grass pea: "Derek". The seeds were obtained from Professor Wojciech Rybiński at the Institute of Plant Genetics of the Polish Academy of Sciences (Poznań, Poland). Explants for protoplast isolation came from *in vitro* and *in vivo* grown seedlings. To obtain seedlings *in vitro*, seeds were surface sterilized for 60 s in 70% ethanol, 25 min in 0.1% $HgCl_2$ and then rinsed five times in sterile distilled water. The seeds were placed on an agar solidified medium for seed germination composed of MS macro and microelements (Murashige and Skoog, 1962) with 20 g/l sucrose. Sterile plant material was cultured at 25 ± 1°C under a 16/8 h light photoperiod of 50 μmol/m/s photosynthetic photon flux density. In order to obtain explants from plants *in vivo*, 6-8 seeds were sown to pots (φ 12 cm) containing universal soil and left in a greenhouse at approx. 18°C. Collected explants (leaves) were sterilized as follows: 60 s immersion in 70% ethanol, 3 min immersion in 0.1% $HgCl_2$ and 5-rinses in sterile distilled water.

Optimization of protoplast isolation

Leaves from 10, 15, 21 and 28 day-old seedlings grown *in vitro* and *in vivo* were used as explants for protoplast isolation. Leaves with the lower epidermis removed were incubated in 10 ml of an enzymatic mixture. Two enzymatic mixtures were used: EL I composed of 2% Cellulase "Onozuka" R-10 (Kinki Yakult), 1% Pectinase (Sigma) and EL II composed of 1% Cellulase "Onozuka" R-10 (Kinki Yakult) and 0.5% Macerozyme (Kinki Yakult). The enzymatic mixtures were prepared in a CPW solution (Frearson et al., 1973) with 0.1% MES [2-(N-morpholino) ethanesulfonic acid] buffer and 11% (*in vitro* seedlings) and 12% (*in vivo* seedlings) sorbitol. In EL I solution, the material was incubated for 3-4 hours with shaking (100-120 rpm). In EL II solution, leaves were incubated overnight (17-18 hours), statically at 26 ± 1°C. Protoplasts were purified by filtration through a 100 μm nylon-mesh filter and afterwards were rinsed three times with an appropriate sorbitol solution and twice with the respective culture medium.

Assessment of the isolation conditions

To evaluate the applied isolation conditions, the efficiency of protoplast isolation (the number of protoplasts per 1 g of fresh weight tissue) and protoplast viability (%) directly after isolation were assessed. Protoplast viability was assessed by staining the protoplasts with an aqueous solution of 0.1% Evans Blue (Gaff and Okong'o-Ogola, 1971). Viable protoplasts remain colorless (the dye does not pass through the plasma membranes), while non-viable protoplasts are stained blue (the dye penetrates through the damaged membranes). These observations were conducted under a light microscope (Eclipse E400, Nikon). Additionally, protoplasts were suspended in liquid B1 medium and their viability was controlled during the subsequent days of culture. Those conditions of protoplast isolation that gave the longest viable protoplasts were used in further protoplast culture studies.

Protoplast culture

Protoplasts were cultivated in 12 different liquid media (Table 1). The density of the culture was 1×10^5 protoplasts/ml. They were then suspended in 2 ml of a medium in Petri dishes (φ 40 mm). The liquid medium of the composition favoring the longest survival protoplast rate was chosen to establish a solid protoplast culture

Table 1. Composition of the media used for grass pea protoplast liquid culture

Medium Code	Mineral salts and vitamins	Organic compounds [mg/l]	Growth regulators [mg/l]
K1	KM[a]	250 fructose, 250 ribose, 250 xylose, 250 mannose, 40 citric acid, 40 fumaric acid, 40 malic acid, 250 casein hydrolyzate, 250 sucrose, 60 000 glucose, 20 ml coconut water[a]	1.0 NAA 0.2 2,4-D 0.5 zeatin[a]
K2	KM	as in K1, 0.1 L-tyrosine, 0.1 L-asparagine, 0.1 L-phenylalanine, 0.1 adenine, 0.1 L-serine, 0.1 L-proline, 0.1 DL-tryptophan	as in K1
B1	B_5[b]	100 MES, 20 ascorbic acid, 10 000 sucrose, 5 000 glucose	0.5 NAA, 0.5 2,4-D, 0.5 2iP, 0.5 BAP, 0.5 GA_3
B2	B_5	as in B1, amino acids as in K2	as in B1
B3	B_5	as inB1, 2.0 chitosan	as in B1
B4	B_5	as inB1, 5.0 chitosan	as in B1
B5	B_5	as inB1, 7.5 chitosan	as in B1
B6	B_5	as inB1, 10.0 chitosan	as in B1
B7	B_5	as inB1, 1 000 Pluronic F-68	as in B1
B8	B_5	as inB1, 10 000 Pluronic F-68	as in B1
B9	B_5	as inB1, 5.0 chitosan, 10 000 Pluronic F-68	as in B1
B10	B_5	as inB1, 50 acetylsalicylic acid	as in B1
B11 *	B_5	as in B4	as in B1
B12 #	B_5	as in B4	as in B1

a – according to Kao and Michayluk, 1975; b – according to Gamborg et al., 1968; * – sodium-alginate culture; # – agarose-droplets culture

using agarose and sodium alginate. For the agarose-droplet culture, a protoplast suspension (2×10^5 protoplasts/ml) was mixed with an equal volume of a double-strength medium with 1.6% (w/v) Sea Plaque agarose (Cambrex Biosciences, Rockland, Maine, USA). The suspension was used to form 8 agarose droplets (each of 125 µl volume) in one Petri dish. After solidification, the droplets were overlaid with 2 ml of B4 liquid medium. For the sodium-alginate culture, the protoplast suspension (2×10^5 protoplasts/ml) was mixed with an equal volume of 2.8% (w/v) sodium alginate (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany). A total volume of 200 µl of the suspension was plated on the agar solidified Ca^{2+}-medium to form thin alginate layers. After solidification, alginate layers were transferred to Petri dishes containing 2 ml of B4 liquid medium. Cultures were incubated in the dark at $26 \pm 1\,^{\circ}C$.

Assessment of culture conditions

The viability of protoplasts was evaluated on the isolation day, and then after 1, 5, 10 and 15 days of culture. At those times, changes in protoplast morphology were also assessed. Protoplasts were categorized as spherical (protoplasts with their original shape), enlarged (more than twice the normal volume), budding (protoplasts with protuberances), oval (non-spherical, egg-shaped), snowmen-like (protoplasts with a narrowing in the equatorial part) and degenerating (darkened, collapsed protoplasts). Additionally, the rate of cellulosic cell wall reconstruction was estimated after 24 hours of culture. The regenerated cell walls of cultured protoplasts were stained with Calcofluor White (Sigma Chemical Co.) (Nagata and Takebe, 1970). A total volume of 100 µl of the protoplast suspension was mixed with an equal amount of 0.01% aqueous dye solution. Protoplasts were stained for 5-10 minutes. After preparing specimens, protoplasts were viewed using a fluorescence microscope (AxioImager M2 multifunctional microscope, Zeiss) under UV light at a wavelength of 365 nm. Photographic documentation was made using the AxioVision Rel. 4.8. program (Zeiss) and a PowerShot G10 camera (Canon).

Statistical analyses

The experiment was performed in three replicates. In the isolation experiment, the replicate was each combination of isolation conditions was tested in replicate. In the culture experiment, the replicate was three Petri dishes of each medium. Protoplast viability, morphology changes and the wall reconstruction of the cell were expressed as percentages. At least 100 randomly chosen protoplasts were calculated in each count. The *Post hoc* LSD test was used to study differences between the means at $P = 0.05$.

Results

Protoplast isolation efficiency

Protoplast isolation efficiency from leaves of *in vitro* grown grass pea seedlings was significantly higher using the EL II enzymatic mixture (Table 2). The age of *in vitro* grown seedlings did not significantly affect the number of released protoplasts (Table 2). However, considering together the donor material age and the type of enzymatic mixture, the highest protoplast isolation efficiency was observed from leaves of 21 and 28 day-old *in vitro* grown seedlings incubated in EL II solution (Table 2), whereas leaves from the oldest seedling incubated in EL I solution gave the smallest number of protoplasts. In the case of leaves collected from *in vivo* grown seedlings, there was no noticeable difference in isolation efficiency between the two applied enzymatic mixtures (Table 2). In contrast, the age of donor plants affected the number of released protoplasts. The highest yield of protoplasts was isolated from 15-day-old seedlings, and the lowest from 28-day-old ones (Table 2). The statistical analysis of these two influencing factors (enzyme mixture and seedling age) showed that most mesophyll protoplasts were obtained from the leaves that were harvested from 15 and 21 day-old *in vivo* grown seedlings, incubated in EL II enzyme mixture. The EL I mixture, containing a higher concentration of enzymes, was the most effective in terms of protoplast isolation from the youngest seedling leaves. Table 2 shows that a higher protoplast isolation efficiency was obtained using the *in vivo* leaves.

Viability of freshly isolated protoplasts

The viability of freshly isolated mesophyll protoplasts obtained from *in vitro* as well as *in vivo* grown plant material depended on the applied enzymatic mixture and was significantly higher after a short period of incubation in EL I solution (Table 2). The age of a donor plant had no impact on the viability of protoplasts isolated from *in vitro* leaves, regardless of the enzymatic mixture solution (Table 2). In contrast, *in vivo* leaves from the youngest and the oldest seedlings released the least viable protoplasts in EL II (Table 2). Taking into account only the origin of the material, there was no difference in the viability of protoplasts directly after isolation (Table 2).

Viability of protoplasts in B1 medium

Generally, the viability of protoplasts isolated from *in vitro* grown material declined significantly more slowly than *in vivo* protoplasts in the following days of culture (data not shown). Protoplasts from greenhouse seedlings degenerated regardless the type of enzyme applied and the age of the donor plant. In turn, *in vitro* protoplast viability noticeably decreased at a much slower pace after an overnight isolation in EL II enzyme, when leaves of 15 or 21 day-old seedlings were used as explants.

Protoplast viability in the culture experiment

In the whole experiment, the viability of freshly isolated protoplasts differed between each replication, so the survival rate of protoplasts was expressed as the percentage increase in the number of dead protoplasts in relation to initial viability. A completely lethal effect on the protoplasts was demonstrated by the addition of chitosan at a concentration above 7.5 mg/l (B5 and B6 media) and by a combination of chitosan and Pluronic F-68 (B9). On the second day after the culture was established, no viable protoplasts were observed in these media (Table 3). Very strong decline in the protoplast viability, after one-day cultivation, was also noted in K1, K2 and B10 media (Table 3). After one-day cultivation, the greatest survival rate was noticed for protoplasts suspended in B1 medium and media enriched with lower concentrations of chitosan (B3, B4) and 10 g/l Pluronic F-68 (B8) (Table 3). After 5 days of culture, the lowest decrease in protoplast viability was recorded in a B4 medium which contained 5 mg/l chitosan. At this length of cultivation, no viable protoplasts were observed in K1, K2 or B10 media. Protoplasts immobilized in a sodium alginate film had the lowest rate of viability decline after 10 days of culture. However, among all the media tested, only in liquid B4 medium were viable protoplasts noted after 15 days of culture (Table 3).

Table 2. Protoplast isolation efficiency (IE) and viability (V) depending on different factors

Explant origin			Enzymatic mixture			Age of donor plant		
	IE ($\times 10^6$) protoplasts/g f.w.	V (%)		IE ($\times 10^6$) protoplasts/g f.w.	V (%)		IE ($\times 10^6$) protoplasts/g f.w.	V (%)
in vitro	4.8 b	58 a	ELI	3.5 b	63 a	10	4.1 b	59 a
						15	4.8 ab	53 a
			ELII	6.2 a	51 b	21	5.5 a	55 a
						28	4.2 b	68 a
in vivo	6.1 a	60 a	ELI	5.0 a	81 a	10	4.6 bc	60 ab
						15	8.0 a	71 a
			ELII	7.1 a	39 b	21	6.9 ab	64 a
						28	3.6 c	36 b

Origin/enzymatic mixture/age of donor plant				Origin/enzymatic mixture/age of donor plant			
in vitro		IE ($\times 10^6$) protoplasts/g f.w.	V (%)	*in vivo*		IE ($\times 10^6$) protoplasts/g f.w.	V (%)
ELI	10	3.2 ef	68 ab	ELI	10	7.6 bc	85.5 ab
	15	3.9 de	55 bc		15	4.6 cd	86 a
	21	4.3 cde	59 abc		21	5.8 bcd	79 ab
	28	2.2 f	77 a		28	3.6 d	70 abc
ELII	10	5.1 cd	47 c	ELII	10	3.1 d	35 cd
	15	5.6 bc	51 bc		15	11.5 a	55 abc
	21	6.8 ab	50 bc		21	8.1 b	49 bc
	28	8.3 a	58 abc		28	3.6 d	2.5 d

Means with the same letter are not significantly different; g f.w. – g fresh weight

Table 3. Increase in protoplast death (%) on subsequent days of culture in different media

M	Day of culture			
	1	5	10	15
K1	90.7 c	100.0 h	100.0 d	100.0 b
K2	94.1 c	100.0 h	100.0 d	100.0 b
B1	9.1 a	71.9 e	96.2 cd	100.0 b
B2	35.6 b	89.7 g	100.0 d	100.0 b
B3	8.3 a	31.7 b	95.7 cd	100.0 b
B4	13.4 a	20.2 a	74.6 b	92.9 a
B5	100.0 c	100.0 h	100.0 d	100.0 b
B6	100.0 c	100.0 h	100.0 d	100.0 b
B7	35.6 b	81.2 f	97.7 cd	100.0 b
B8	16.3 a	66.9 e	90.3 c	100.0 b
B9	100.0 c	100.0 h	100.0 d	100.0 b
B10	94.9 c	100.0 h	100.0 d	100.0 b
B11	14.7 a	44.7 c	64.0 a	100.0 b
B12	7.0 a	51.9 d	71.1 b	100.0 b

Means (within one time of observation) with the same letter are not significantly different

Protoplast morphology changes

Generally, after one day of culture no changes in the protoplasts shape were observed in the majority of media used. Only in media containing Pluronic-F68 were a small percentage of budding protoplasts observed (Table 4). The percentage of spherical protoplasts with regularly arranged bright-green chloroplasts varied depending on the media (Fig. 1B). Most spherical protoplasts (65.1%) were noted in liquid B4 medium (Table 4). During the subsequent culture days, some changes in protoplast morphology (oval and snowmen-like protoplasts) were recorded, evidencing cell preparation to mitotic division (Fig. 1C and 1D). The majority of these protoplast types were observed after 5 days of cultivation in a liquid medium containing 5 mg/l chitosan and after 10 days in an agarose-droplet culture (8.2%) (Table 4). Despite further control of the culture reactions, no steady, repeated mitotic activity of cells was recorded. Nevertheless, sporadic, single divisions were observed in the agarose-droplet culture (Fig. 1H and 1I), while protoplast "budding" occurred very often (Table 4, Fig. 1F). A common phenomenon was the occurrence of strongly enlarged protoplasts, with a diameter of 2-3 times greater than the average (Table 4, Fig. 1E). Changes in protoplast morphology mostly occurred in the first week of cultivation, and the addition of chitosan, apart from prolonging the viability of the protoplasts, stimulated protoplast budding relative to the B1 medium and addition of the Pluronic F-68 (10 g/l) limited this process (Table 4).

Protoplast cell wall reconstruction

Grass pea protoplasts rebuilt their cell walls with different frequencies depending on the media composition (Fig. 1G and Fig. 2). After 24 hours, more than half (58-61%) of cultivated protoplasts deposited cell walls in B1, B2, B3 and B4 media. In the media that do not favor the survival of protoplasts, the rate of cell wall regeneration was low (10-17%). An addition of Pluronic F-68 to the media and embedding of protoplasts in solid media slowed down the process of cell wall reconstruction.

Discussion

The procedures of protoplast isolation, its culture and complete plant regeneration have been developed for many plant species (Davey et al., 2005). However, the protoplast culture technique, with its necessity of very precise treatments on fragile explants, is regarded as the most difficult of all *in vitro* methods (Lal and Lal, 2000). Additionally, legume plant species are considered to be very recalcitrant to *in vitro* conditions (Rybczyński, 2001). The present studies were undertaken to optimize the isolation conditions of grass pea "Derek" protoplasts and elaborate the initial stages of their further culture.

To a large extent, protoplast isolation efficiency is affected by the type of explant and the age and physiological condition of the donor plants (Babaoglu, 2000; Sinha et al., 2003). Mesophyll protoplasts are characterized by homogeneity and genetic stability (Blackhall et al., 1994) and have been successfully used to regenerate many plant species (Takebe et al., 1971; Shepard and Totten, 1977; Atanassov and Brown, 1984; Russell and McCown, 1988; Hu et al., 1999; Thomas, 2009; Castelblanque et al., 2010). In the present experiments, isolation from leaf mesophyll of grass pea seedlings gave a sufficient number of protoplasts suitable for further culture research. In isolation optimization procedures for other legumes, a clear correlation between the age of donor plant and isolation parameters have been demonstrated (Gill et al., 1987; Babaoglu, 2000; Sinha et al., 2003; Wiszniewska and Pindel, 2013). In the present experiment, the best result for the isolation parameter was achieved when leaves originating from 15 and 21 day-old seedlings were used.

In our study, enzymatic mixtures comprising the enzyme with cellulolytic (2% or 1% Cellulase "Onozuka" R10) and pectinolytic activity (1% Pectinase or 0.5% Mecerozyme) were used. McCutchan (2001) for the isolation of grass pea protoplasts, from shoot tips and young leaves, as well as cell suspensions used a mixture containing four different enzymes (1% Cellulysin, 0.5% Driselase, 0.5% Pectinase and 0.1% Pectolyase Y23). On the other hand, Durieu and Ochatt (2000) isolated protoplasts from an embryonic shoot axis using a mixture of 4% Cellulase Onozuka R10, 3% Macerozyme and 0.2% Pectolyase Y-23. In the present experiments, the best protoplasts isolation efficiency reached 11.5×10^6 protoplasts /g fresh weight) and was much higher than the results obtained by McCutchan (2001) – 3×10^6 protoplasts/g f.w., as well as Durieu and Ochatt (2000) – 2×10^6 protoplasts /g f.w. The process of protoplast isolation is an important step in the procedure of plant regeneration from protoplast culture. It induces stress that affects a subsequent response of protoplasts to cult-

Table 4. Protoplast morphology changes (%) on subsequent days of culture in different media

M	Day of culture																
	1			5						10					15			
	S*	D	B	S	D	E	B	O	SN	S	D	E	B	O	S	D	B	O
K1	5.4 f	94.6 e	0 b	0 e	100 g	0 e	0 d	0 f	0 c	0 e	100 g	0 d	0 b	0 e	0 b	100 b	0 b	0 b
K2	3.4 f	96.6 e	0 b	0 e	100 g	0 e	0 d	0 f	0 c	0 e	100 g	0 d	0 b	0 e	0 b	100 b	0 b	0 b
B1	54.1 bc	51 bc	0 b	7.8 cd	75.9 d	9.7 a	4.5 b	1.1 e	1.1 a	0 e	94.6 ef	1.0 cd	3.9 b	0.8 cde	0 b	100 b	0 b	0 b
B2	28.8 e	72.2 d	0 b	3.0 de	88.3 f	2.2 cd	2.9 bc	2.2 d	1.4 a	0 e	100 g	0 d	0 b	0 e	0 b	100 b	0 b	0 b
B3	52.7 c	47.3 bc	0 b	4.1 de	79.5 de	0.8 de	10.7 a	4.9 c	0 c	1.9 de	87.6 d	5.7 a	3.8 b	1.0 cd	0 b	100 b	0 b	0 b
B4	65.1 a	34.9 a	0 b	33.7 a	40.5 a	4.4 bc	12.5 a	8.5 a	0.4 b	6.4 c	67.7 a	5.4 ab	18 a	2.0 bc	0.5 a	89.5 a	8.4 a	0.4 a
B5	0 f	100 e	0 b	0 e	100 g	0 e	0 d	0 f	0 c	0 e	100 g	0 d	0 b	0 e	0 b	100 b	0 b	0 b
B6	0 f	100 e	0 b	0 e	100 g	0 e	0 d	0 f	0 c	0 e	100 g	0 d	0 b	0 e	0 b	100 b	0 b	0 b
B7	40.1 d	56.9 c	2.0 a	6.4 cde	84.5 ef	3.3 c	4.5 b	1.3 e	0 c	1.5 de	98.6 fg	0 d	0 b	0 e	0 b	100 b	0 b	0 b
B8	55.3 abc	44.3 ab	0.3 b	12.3 c	77.9 d	6.9 b	1.7 cd	1.3 e	0 c	3.8 cd	92.2 de	2.7 bc	1.0 b	0.5 de	0 b	100 b	0 b	0 b
B9	0 f	100 e	0 b	0 e	100 g	0 e	0 d	0 f	0 c	0 e	100 g	0 d	0 b	0 e	0 b	100 b	0 b	0 b
B10	3.7 f	96.1 e	0 b	0 e	100 g	0 e	0 d	0 f	0 c	0 e	100 g	0 d	0 b	0 e	0 b	100 b	0 b	0 b
B11	56.9 abc	43.1 ab	0 b	36.2 a	63.2 b	0 e	0 d	0.7 ef	0 c	21.5 a	76.0 b	0 d	0 b	2.5 b	0 b	100 b	0 b	0 b
B12	62.0 ab	38.0 a	0 b	23.7 b	67.8 c	2.5 cd	0 d	5.9 b	0 c	10.1 b	80.8 c	1.0 cd	0 b	8.2 a	0 b	100 b	0 b	0 b

* S – spherical; D – degenerating; E – enlarged; B – budding; O – oval; SN – snowmen-like protoplasts

Means (within one protoplast type and time of observation) with the same letter are not significantly different

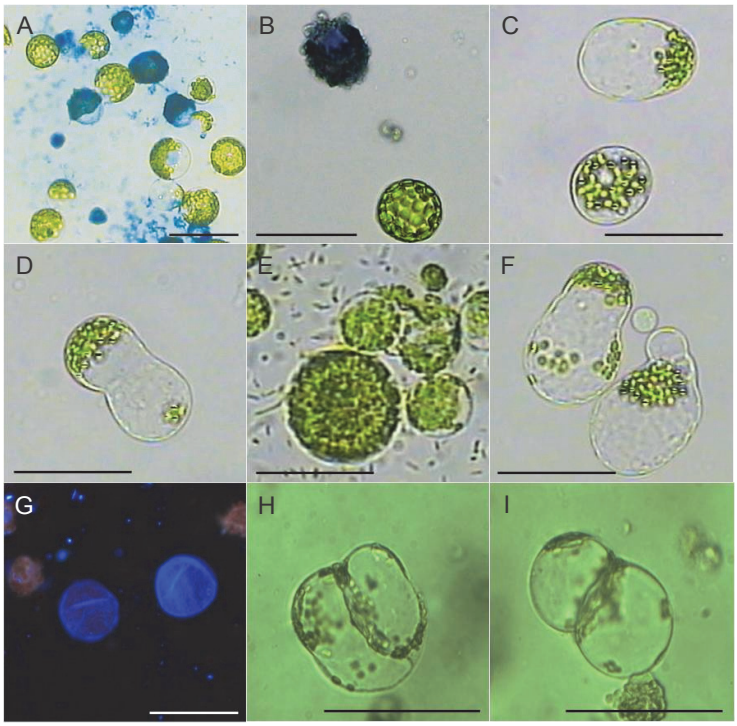

Fig. 1. Protoplasts of grass pea A-B) viability assessment of freshly isolated protoplasts (blue – non-viable protoplasts); C-F) protoplast morphology changes C) oval, D) snowmen-like, E) enlarged, F) budding protoplasts; G) visualization of reconstructed cell wall after 24 hours of cultivation; H-I) single cell division in agarose-droplet culture. Bar – 50 μm

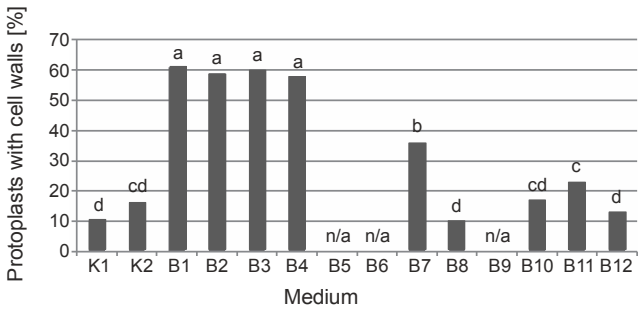

Fig. 2. Percentage of protoplasts with regenerated cell walls after 24 hours in different media; means with the same letter are not significantly different

ture conditions (Papadakis et al., 2001; Papadakis and Roubelakis-Angelaskis, 2002). The selection of a specific enzyme mixture influences not only the number of released protoplasts, but also their further morphogenic reactions in culture. Ochatt et al. (2000) observed that the application of an enzyme mixture different from that most suitable for a particular pea genotype resulted in an almost total lack of protoplast divisions.

In the present study, material incubated for 3-4 hours gave more viable protoplasts, although it gave a lower protoplast yield than during a prolonged incubation time. A short digestion period also reduced the yield of broad bean protoplasts, but its extension to over 16 hours decreased protoplast viability (Tegeder et al., 1995). Despite these findings, we decided to use overnight incubation and the ELII solution to isolate protoplasts for culture studies. This decision was motivated by the observation that, despite higher protoplast viability immediately after a short rather than overnight incubation, protoplasts from a short incubation degenerated much faster on subsequent days of culture. This most likely resulted from mechanical damage generated during shaking. The overnight incubation procedure has also been preferred in numerous studies on the protoplast culture of legumes (Puonti-Kaerlas and Eriksson, 1988; Rozwadowski et al., 1990; Tegeder et al., 1996; Durieu and Ochatt, 2000; Ochatt et al., 2000; McCutchan, 2001). The highest viability of grass pea protoplast directly after isolation was approx. 86%, which was similar to the results obtained by Durieu and Ochatt (2000).

Protoplasts originating from different species and even from various tissues of the same plant vary in their requirements for culture conditions, especially with regards to the medium composition. The optimal composi-

tion needs to be established experimentally (Davey et al., 2005). Between the compared two basal media (B_5 and KM), KM proved to be ineffective for the culture of grass pea mesophyll protoplasts. Similarly, McCutchan (2001) noted no reaction of grass pea protoplasts to this medium. On the other hand, Durieu and Ochatt (2000) obtained the highest frequency of divisions leading to microcalli formation on a KP medium, i.e. a KM medium containing 0.1 mg/l of 2,4-D, 0.2 mg/l zeatin and 1 mg/l NAA. In our study, better results – extended protoplast viability – were recorded on a modified B_5 medium. A modified B_5 medium also stimulated divisions of *L. odoratus* protoplasts (Razdan et al., 1980). The supplementation of media with substances having a positive effect on different plant species protoplast cultures – Pluronic F-68, acetylsalicylic acid (Carswell et al., 1989; Anthony et al., 1994) – did not stimulate grass pea protoplasts to divide. Instead, the longest viability of protoplasts was observed in media enriched with chitosan, which is a rather new compound tested in tissue culture systems (Uddin et al., 2004; Smith et al., 2006; Uthairatanakij et al., 2007; Limpanavech et al., 2008). The immobilization of protoplasts using agarose or sodium alginate has a beneficial effect on the induction of protoplast division, development of cell colonies and callus formation in many plant species (Shilito et al., 1983; Pati et al., 2005; Pati et al., 2008). Agarose embedding also caused a significant enhancement of the division rate in recalcitrant yellow lupine protoplasts (Wiszniewska and Pindel, 2009). However, in the described grass pea culture only single, sporadic divisions were observed in an agarose-droplet culture in B4 medium.

Up to 18% of grass pea protoplasts underwent "budding" in the established cultures. An incomplete or weak regeneration of the cell wall is believed to be the main reason for this process (Horine and Ruesink, 1972; Meyer and Abel, 1975; Firoozabady and DeBoer, 1986). The increasing frequency of "budding" was also attributed to the excessive protoplast density (Russell McCown, 1988) and a drastic pH decrease in the medium (Lehminger-Mertens and Jacobsen, 1989). In turn, Babaoglu (2000) observed a more frequent "budding" of enlarged *Lupinus mutabilis* protoplasts. After 24 hours, from 10% to 60% of "Derek" grass pea protoplasts created a new cell wall depending on the medium composition. In contrast, after two days of culture only 7-9% of mesophyll yellow lupine protoplasts had visible cell walls (Wiszniewska and Pindel, 2013). However, the presence of the previously mentioned "budding" points indirectly to irregularities in the reconstruction of cell walls, which can affect the mitotic activity of protoplasts. Total cell wall regeneration determines the cell mitotic activity (Nagata and Takebe, 1970; Tylicki et al., 2001).

Conclusions

A rapid decline in protoplast viability, together with disturbances in cell wall rebuilding are the main reasons for impaired development of grass pea protoplast cultures. Nevertheless, the first protoplast division observed in an agarose solidified medium indicates that immobilization is necessary to stimulate further culture growth. The present results confirm that grass pea protoplasts are difficult to study. However, the high utility potential of this species and the prospect of achieving somatic hybridization that may induce a new genetic variability encourages attempts to search for new methods enabling further development of this *in vitro* technique.

Acknowledgements

This study was financially supported by the Polish Ministry of Science and Higher Education (DS 3500).

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BioTechnologia vol. 96(2) • pp. 203-207 • 2015
Journal of Biotechnology, Computational Biology and Bionanotechnology SHORT COMMUNICATION

Establishment of an *in vitro* culture of *Pelargonium* × *domesticum* cultivars characterized by different growth requirements

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Abstract

Experiments were carried out to develop micropropagation protocols for cultivars of hybrid origin which have been not cultivated *via* tissue culture. Proliferating cultures of *Pelargonium* × *domesticum* "Tip Top Duet" and "Black Knight" were obtained even if initially the number of excised aseptic nodal pieces was considerably reduced due to contamination with endogenic bacteria. The supplementation of a maintenance medium with either 100 or 200 mg/l peptone proved beneficial. The best propagation coefficient, exceeding 82 adventitious shoots from one initial microcutting, was obtained on a modified MS medium with an addition of 200 mg/l peptone and 0.5 mg/l adenine. The regenerated shoots readily developed new, anatomically properly formed leaves. In the plant material studied, the leaf epidermis produced glandular trichomata which were similar in structure to those observed in a standard cultivar.

Keywords: micropropagation, *Pelargonium*, plant tissue culture, Geraniaceae, peptone

Abbreviations
BA – benzyladenine
IAA – indole -3-acetic acid
IBA – indole-3-butyric acid
NAA – 1-naphtaleneacetic acid
PPFD – photosynthetic photon flux density

Introduction

The section *Pelargonium* L. Hér. (Geraniaceae) containing both aromatic and non-aromatic species is a typical element of the Cape Floristic Region. According to the findings of floristic studies, the family Geraniaceae is one of the most commonly represented in the region, in terms of the number of recognized taxons. Genus *Pelargonium* comprises about 250 species, of which almost sixty percent are endemic to Africa. About 160 species that belong to the Flora of Southern Africa (FSA) region have a wide distribution throughout the FSA area (van der Walt and Vorster, 1983; Bakker et al., 1999, 2000; Weng et al., 2012; Martinez-Cabrera and Peres-Neto, 2013). Specimens of numerous taxa are conspicuously tomentose, and frequently aromatic. Their leaves come in a variety of shapes and sizes. Some *Pelargonium* species, and their derived cultivars with beautiful five-petalled flowers – widely known as rose-scented geraniums or regal geraniums – are eagerly cultivated as popular indoor or outdoor ornamental plants (Mosina et al., 2014; Darras et al., 2015). Several other species from this genus are also valued as medicinally important plant materials (Saraswathi et al., 2011; Moyo and Van Staden, 2014). Extracts obtained from some of these are characterized by a high level of antimicrobial activity (Lis-Balchin and Deans, 1996). Numerous *Pelargonium* species are especially known as commercially important essential oil yielding aromatic crops (Rao et al., 1996; Rao, 2002; Nejad and Ismaili, 2013).

In commercial production, new cultivation technologies and healthy starting materials are of crucial importance (Murthy et al., 1996; Wojtania et al., 2004; Milošević et al., 2012). An *in vitro* culture provides an ideal opportunity to overcome those factors which could limit efficient propagation. Thus, mass production of the highest quality plant material is currently possible due to the

exploitation of appropriate plant tissue and cell culture techniques. Some common approaches to *in vitro* regeneration technology for the members of genus *Pelargonium* have previously been described, including caulogenesis, somatic embryogenesis and formation of embryo-like structures (Mithila et al., 2001; Madden et al., 2005). Additionally, previous experiments have revealed that each genotype under study usually requires specific culture media components to regenerate effectively (Quresji and Saxena, 1992; Desilets et al., 1993; Wojtania and Gabryszewska, 2001). Moreover, particular genotypes respond differently to the type of plant growth regulators or their analogs supplemented to the medium. This is especially true for substances with cytokinin-like activity, as well as for their combinations with auxins or auxin-like compounds (Madden et al., 2005). Thus, it is of utmost importance to elaborate an *in vitro* culture protocol to induce and sustain the regeneration capacity of specific *Pelargonium* species, subspecies or cultivars. The goal of this study was to establish a tissue culture of the precious and attractive *Pelargonium* × *domesticum* cultivars. We focused, in particular, on obtaining a proliferative shoot culture of regal geranium and, consequently, healthy looking microcuttings ready to be rooted. The regenerated shoot leaf epidermis and anatomical features of newly obtained leaf blades were studied and compared. Our objective was to evaluate the effectiveness of the applied regeneration protocol and to gather some experimental data for future taxonomical comparisons.

Materials and methods

The plant material was obtained from the Botanical Garden in Prague (Botanická Zakrada hl. m. Prahy, Czech Republic). *Pelargonium* cultivars of hybrid origin obtained on the basis of *Pelargonium grandiflorum* which was kindly provided by Dr. Stolarczyk (University of Agriculture in Krakow) were used as donors to initiate the tissue culture. Plant material for excision of the primary explants represented *Pelargonium* × *domesticum* "Tip Top Duet" and "Black Knight" cultivars. The "Tip Top Duet" cultivar prefers sunny stands. It blooms from June to August, its flowers are two-colored, violet and pink, with a velvet appearance to its bright green leaves. "Black Knight" prefers shady stands, blooms from April to September, and its two-colored petals are dark violet and white. Its leaves are dark green.

In vitro culture initiation

First, a procedure for the surface disinfection of stem pieces was elaborated. Next, in order to initiate a culture from both studied cultivars, 40 primary explants were excised, with two explants constituting a single replicate. Primary explants for culture initiation were taken from the *Pelargonium* collection maintained under greenhouse conditions. Nodal segments about 10 mm long with lateral buds excised from young stems were surface sterilized by immersing in 70% ethanol (60 s), followed by immersion in 0.1% (v/v) mercuric chloride solution containing Tween-80 (2 drops per 100 ml) (2 min). Explants were then thoroughly rinsed 5 times in sterile distilled water. Later, explants were transferred into initiation media E and E_C, which were composed of Murashige and Skoog mineral salts (1962) with doubled content of amino acids, and the media was supplemented with 30 g/l sucrose. If required, the initiation media contained 0.05 mg/l indole-3-butyric acid (IBA) and 0.25 mg/l benzyladenine (BA) g/l, and were solidified with 0.8% Difco agar (Difco Laboratories Inc., UK). Additionally, the E_C medium contained 0.6 g/l activated charcoal. Medium pH was adjusted to 5.8 prior to autoclaving at 0.122 MPa for 20 min. The shoots that developed were subcultured in the same medium. The cultures were indexed for growth and the possible mortality of explants due to visible bacterial contamination.

Stage of vegetative propagation

Shoots developed on the initiation media (E/E_C) were transferred onto the propagation media (P_K, P_1, P_2) listed in Supplementary Table 1. The main differences between particular treatments lay in the possible application of peptone, adenine, and plant growth regulators as well as in their dose. Secondary explants were taken from aseptic shoot cultures of respective cultivars which had been maintained in *in vitro* conditions. Such explants were 10 mm long apical shoot parts. Subculturing of cultivated organs was performed at four-week intervals. The culture was regularly documented. Simultaneously, basic biometrical data and anatomical features were recorded. Microscopic observations of regenerated leaf epidermis were performed with a light microscope (Nikon Eclipse 400), according to the procedure described elsewhere (Stolarczyk et al., 2013). The upper and lower epidermis was removed from the leaf blade and

analyzed in relation to the type, structure, and length of trichomata. Calculations were made using Image Pro-Plus 4.0 software. The propagation coefficient, calculated as the number of new shoots developed from a single secondary explant, was assessed at the end of every passage.

Growth conditions and statistical analysis

Explants were cultured in 100 ml Erlenmayer flasks filled with 25 ml of culture medium. Cultures were maintained in a growth chamber, at the day temperature of 25°C, and the night temperature of 20°C, with a 16/8 photoperiod. The culture environment was maintained at 80 μmol/m^{2}s of PPFD. Cool white fluorescent lamps were used as a light source. Experiments were organized according to a randomized design with 40 explants per treatment and two repetitions of the experimental unit. The results were subjected to a statistical analysis using the ANOVA module in STATISTICA ver. 10.0 software. A post-hoc Tukey test was used to study the differences between the means at $P < 0.05$.

Results and discussion

During culture initiation, axillary buds of excised nodal segments slowly developed into shoots. However, the total number of regenerated shoots was reduced due to contaminations with latent bacteria, which was mainly apparent during the first passage (Supplementary Table 2). During the course of the initial culture, the activated charcoal supplementation did not influence either the quality or the regeneration efficiency of the shoot. Nevertheless, the number of viable explants was sufficient to initiate the culture (Supplementary Fig. 1) and to develop new leaves from buds. The aim of the next stage was to obtain a proliferative culture of regal geranium. Culture media used in the experiment (Supplementary Table 1) appeared to be appropriate to obtain successfully proliferating shoot culture. The regeneration efficiency proved to be similar for all media tested during the first passage, but varied in the successive passages (Supplementary Table 3). Microshoots obtained in every passage were intensely green, up to several millimeters high, and usually formed two or three new leaves (Fig. 1). As shown in Supplementary Table 3, the regeneration efficiency of *Pelargonium* × *domesticum* "Tip Top Duet" was slightly elevated in comparison to "Black Knight". During the proliferation stage, an addition of peptone to the medium was beneficial for the formation of new axillary buds, especially for the "Tip Top Duet" cultivar, even though after 14 days of culture the propagation coefficient was 21.5 for "Black Knight" and 22.2 for "Tip Top Duet". It was ascertained that the addition of 200 mg/l of peptone and 0.5 mg/l adenine to the culture medium resulted in a beneficial effect on the *Pelargonium* × *domesticum* "Tip Top Duet" culture. The highest propagation coefficient, exceeding 82 adventitious shoots from a single secondary explant, was obtained on a modified MS medium supplemented with 200 mg/l peptone and 0.5 mg/l adenine. Regenerated microshoots easily developed new leaves during the elongation stage. Fully expanded new leaves were analogical to those of a standard mother plant, with no spots or color changes. Apart from their small size, *Pelargonium* shoots resembled donor plants grown in greenhouse conditions (Supplementary Fig. 2). Leaves were anatomically properly formed and, especially in the lower epidermis, numerous trichomata were present. Those trichomata which were grandular consisted of two basal cells in the "Black Knight" cultivar and three in "Tip Top Duet" with clearly enlarged basal cells connected with the epidermis. The mean height of fully developed grandular hairs of *Pelargonium* × *domesticum* "Tip Top Duet" was 130 μm and in "Black Knight" 124 μm, whereas non-glandular, unicellular hairs were 327 and 279 μm, respectively.

Fig. 1. Proliferation of *Pelargonium* "Tip Top Duet" shoots on modified MS proliferation medium without peptone (left) and with 100 mg/l of peptone (right)

During culture initiation, some losses of studied cultivars due to bacterial contamination were observed. This is not unusual with cultures of *Pelargonium* and other taxa cultivated *in vitro.* For example, the results of an analysis conducted by Barett and Cassels (1994) revealed that endogenic infection with *Xantomonas campestris* pv. *pelargonii* could be indexed in explants taken

from cultivars of *Pelargonium* × *domesticum.* Sekhar and Thomas (2015) isolated *Fusarium oxysporum* f. sp. *cubense* from shoot-tips of a banana cultivar. Only a few cultivars of *Pelargonium* × *domesticum* have been multiplied vegetatively in a tissue culture (Dunbar and Stephens, 1991; Vejsadová and Kuchtová-Jadrná 2009), and, in the current paper, in "Black Knight" and "Tip Top Duet". Articles published so far have enumerated the following cultivars of *Pelargonium* × *hortorum*: "Bargpalais", "Holywood Red", "Grand Prix", "Jazz Rocky Mountain", "Orbit White", "Scarlet Orbit", "Red Elite", "Ringo Rose", "Ringo Salmon", "White Rocky Mountain", and *Pelargonium* × *hederaefolium* – "Beach", "Bonete", "Luna", "Sofie Cascade", and such like (Desiltes et al., 1993; Murthy et al., 1996; Mithila et al., 2001; Wojtania and Gabryszewska, 2001; Wojtania, 2010; Wojtania et al., 2004; Madden et al., 2005), with petals having many colors, different foliage types and growth habits. In our experiments, we focused on the elaboration of a convenient propagation medium for *Pelargonium* × *domesticum*. Differences in trophic requirements at different stages of *in vitro* culture were shown. Further, when *in vitro* shoot fragments of studied cultivars were transferred onto media supplemented with peptone, organogenesis was prompted, especially in the "Tip Top Duet" cultivar. Peptone is frequently used as an additive in orchid tissue culture (Chen et al., 1999; Kaur and Bhutani, 2012 and herewith cited literature) with a beneficial effect on culture proliferation and morphogenetic response. This diffusible, soluble substance, obtained as a result of a partial hydrolysis of animal protein, is rich in amino acids and has proved to have a positive effect on other plant material, for example banana (Pervin et al., 2013) or purple foxglove (Hagimori et al., 1982). An important feature of the studied culture has been the dimensions of cells and the visual appearance of epidermis. The results of such observations were for the first time conducted in two *Pelargonium* × *domesticum* cultivars – "Black Knight" and "Tip Top Duet" – both valuable ornamental plants. It has been found that the structure of trichomata can be used as a taxonomic tool in systematic comparisons.

Acknowledgements

This study was financially supported by the Polish Ministry of Science and Higher Education in the form of DS 3500/ZBiFR. The financial support is gratefully acknowledged by the authors.

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