

FOCUS ON EUROPE 2021

Journal for breeders and producers of plant material

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- Description of varieties

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Naktuinbouw
+31 (0)71 332 6262
info@naktuinbouw.com
www.naktuinbouw.com



EDITORIAL ADVISORY BOARD

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PUBLISHER/CHIEF EDITOR

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SUB EDITOR Mireille McNutt

GRAPHIC DESIGN Marcel Bakker, De Ontwerperij

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ADDRESS Blue Bird Publishers vof

Jan Kostelijkaan 16
1981 cc Velsen-Zuid
the Netherlands
phone +31 (0)255 521 852
info@bluebirdpublishers.com

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On the cover: The orange Mandarin in the Perfectunia series contains the A1DFR gene. It has been bred for fast and early production with perfect uniformity (Photo: PAC Elsner / Westhoff – www.pac-elsner.com / www.westflowers)

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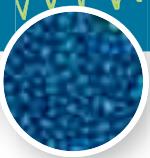
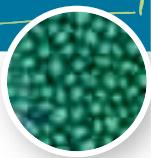


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From Weed to Vegetable Study reveals Caucasian descent

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In Short

New editions Lists of Names

THE NEW EDITIONS (2021-2025) of the List of Names of woody plants and the List of Names of perennials are available. They are preferred names, synonyms and commercial names of almost 45,000 woody plants and 30,000 perennials, and they are the standardised commercial names of the whole range of nursery stock and perennials in Europe and perennials in the United States. The most important changes are:

- Addition of more than 17,000 new names (8,200 in woody plants and 8,800 in perennials)
- Various taxonomic changes in names after consultation with trade and industry

– The status of protection by Plant Breeders' Rights has been added to around 4,000 more cultivars The List of Names of woody crops is supervised by the European Nursery stock Association (ENA), and that of perennials by the Internationale Stauden-Union (ISU). This makes them the international guiding lists in the nursery industry for determining the correct spelling and the preferred names of deciduous trees, conifers, fruit and perennials. The cooperation with these parties ensures a good balance of scientific and legal correctness and practical workability. The Lists of Names are composed by Naktuinbouw, Netherlands. Since 2014, Naktuinbouw has been operating a documentation system of name registration of varieties in nursery stock and perennial crops, and working closely with other registering and coding parties, in particular Varb and Floricode. The new names are mainly notified directly by growers, members of ENA and ISU, or are taken up via the inspection system of Naktuinbouw.

More information The database can be consulted at www.international-plantnames.com. Information on ordering books or a digital version is also available via this website.

Onion genome finally reveals its secrets

Breeding new onion varieties will be

RESEARCHERS FROM Wageningen University & Research have unravelled the onion genome. The onion genome is huge: about sixteen times larger than the tomato genome, and five times larger than that of humans. "Piecing the onion genome together is comparable to completing a puzzle with 100,000 pieces, of which 95,000 are just bits of blue sky," says plant breeding researcher Richard Finkers. "Only 5,000 pieces really make the difference. We managed to sequence a large proportion with the help of the latest DNA sequencing technologies because, with that technology, we managed to assemble the small and large pieces that partially overlap."

Wageningen University & Research collaborated with three companies on sequencing the onion's DNA: the plant breeding companies Bejo Zaden and De Groot en



easier now that the genome is available

Slot, and the genomics company Servicexs. Plant breeders expect that access to the onion genome sequence will double the speed of their breeding work, in future bringing it down to about six or seven years. "In other crops, such as potatoes and rice, the role that certain genes play in providing resistance to disease or tolerance to drought has already been thoroughly studied. Based on the sequence information from genes in those crops, plant breeders can now more easily identify similar genes in the onion genome and more accurately make progress in their own breeding work."

Researchers argue for broadening social debate on patenting

PROTECTION OF INTELLECTUAL property of breeders can be used to ensure that crops are grown according to good agricultural practice and, thus, to more sustainable food production, argue researchers from Wageningen University & Research (WUR). The debate around plant-property patents currently focuses mainly on the risks of monopolies of large multinationals and their possible adverse effects on food systems. This debate can be usefully broadened to include exactly what the opportunities are for making agricultural chains more sustainable.

In a recently published paper in *Agronomy* (MDPI), WUR researchers René Smulders, Clemens van de Wiel and Bert Lotz investigated the compatibility of systems to protect the intellectual property of breeders and make agriculture more sustainable. The debate about the advantages and disadvantages of innovative breeding techniques is now focused on, among other things, the fear that multinationals can abuse patents to build up a dominant position. The authors argue that broad access to new technologies and knowledge must be ensured. At the same time, they argue in favour of the opportunities that intellectual property of innovative breeding techniques can offer.

Editorial

Festive orange

There is nothing better than colourful flowering plants to brighten up your day and, as a Dutchy, orange is by far my favourite. Not only because the orange pennant is often used together with our national flag, but also because our sports teams (and the 'orange army' as their fans are called) usually wear orange shirts/hats/decorations. No wonder that orange makes people from the Netherlands feel festive, particularly in an Olympic year and for football, ice skating or Formula 1 racing supporters.

It was therefore a happy moment when the US government decided to allow marketing of the maize-gene containing bright orange petunias from the German plant breeder Westhoff. On the downside, the application for deregulation of maize-gene containing petunias was an action by only one company and its two orange-loving breeders. It would have been an excellent opportunity to show the world that, if necessary, breeders will stand firmly together. There is also logic – in 2017, many petunia breeders used to have beautiful transgene orange varieties in their collection. Unfortunately, these brightly coloured varieties all had to be destroyed. Petunia breeders will have a second chance though as, so far, only the orange-loving people across the Atlantic can profit from this breakthrough. It does not seem too complicated to convince the European authorities that an A1DFR-gene containing petunia is harmless. Hopefully, this time the bedding plant specialists will cooperate in this matter.

There are many examples of how plant breeders unite for the benefit of all. Take, for instance, the International Licensing Platform Vegetable. Thanks to cooperating and establishing an organization, 15 plant breeders now have easy access to over 250 patented traits, while paying no more than a fair price for it. In the past seven years it has proven its worth. No wonder the breeders of agricultural crops want to follow this example by starting their own platform: the Agricultural Crop Licensing Platform. Hopefully, the future members will be as successful as their vegetable breeding colleagues.

Although petunia breeder Westhoff showed that with enthusiasm and perseverance, one can be successful, the ancient Greek slogan 'United we stand, divided we fall' has not lost its significance.

Monique Krinkels

In Short

List of invasive alien species of EU concern

SINCE 2016, THERE HAS BEEN a list of invasive alien species of EU concern (the so-called 'Union List'). The species included on this list are subject to restrictions and measures set out in the Regulation (EU) 1143/2014. The list now contains 35 plant species, among which are *Eichornia crassipes* (water hyacinth), *Heracleum mantegazzianum* (giant hogweed) and *Impatiens glandulifera* (Himalayan balsam).

"Until now, the consequences for the plant material and breeding sector were not very high. Most plant species on the list are not of high economic importance," explains Marco Hoffman, taxonomist at Naktuinbouw. The most relevant species is *Pennisetum setaceum*. Fortunately, as a result of taxonomical research carried out by the Dutch NVWA (Food and Consumer Product Safety Authority) and Wageningen University, it appeared that the most

important varieties (of which five were protected by EU Plant Breeders' Rights) were classified incorrectly and the botanical name of these five varieties has officially changed from *Pennisetum setaceum* to *P. avena*. This latter species is not on the Union list and has no growing restrictions. Currently, a number of new species are being investigated to decide whether they will be added to the Union List. Some examples are *Pista stratiotes* (water lettuce), *Celastrus orbiculatus* (staff-vine), *Fallopia spp. Broussonetia papyrifera* (paper mulberry) and *Cortaderia selloana* (pampas grass). "Especially the latter causes huge concerns for the perennial plant sector, as it is an economically important crop (millions of plants sold annually). In this crop, around twenty varieties are PBR protected." It is expected that the decision on a new list will be taken by the EU by the end of 2021 or early 2022.

Plant breeders' rights extended

IN ORDER TO encourage innovation, the period covered by Community plant variety rights for the species asparagus, flower bulbs, woody small fruits and woody ornamentals will be extended from 25 years to 30. That is necessary in order to ensure that the investments made in breeding a new plant variety can be recouped

through the resultant earnings. For trees, vines and potatoes, protection periods already have been extended to 30 years. Earlier this year, a vast majority of the EU Committee on Agriculture and Rural Development voted in favour of the proposal. The final decision will be taken by the European Parliament later this year.

Behaviour analysis of insects



The unlocking of behavioural details that have often gone unnoticed will in future lead to more insight into the environmental and genetic mechanisms that control it

NOLDUS INFORMATION

Technology has introduced an automated screening of insect behaviour. It has been developed together with Wageningen University & Research. EntoLab revolutionizes plant breeding through the automated identification of resistance traits against sucking insects, such as thrips, aphids, leafhoppers and whiteflies. Breeding for host-plant resistance to sucking insects has gained much interest in recent years as it represents by far the most economical and ecological solution for farmers. A crucial element in the breeding process is the accurate estimation of the resistance level of large populations of plant accessions or crosses. This requires robust phenotyping systems that can accurately screen many different plant lines with insects in a high-throughput manner. Conventional phenotyping methods focus on costly, labour-intensive and time-consuming

end-point measurements of feeding damage or insect performance, that are often poorly reproducible. The integrated hardware/software system of EntoLab includes a variety of multi-arena leaf holders, special optics, a high-resolution digital video camera, LED illuminators and software for video tracking, data processing and statistical analysis. It has been validated for different species of thrips, aphids and whitefly on pepper, tomato, water melon, chrysanthemum, white cabbage, lily, lettuce and bitter gourd. Evaluations are currently being extended to field crops, such as rice and maize, and to caterpillars and plant hoppers. An example of successful application of the EntoLab system is the work of Dr. Karen Kloth of Wageningen University that led to the identification of the novel aphid resistance gene *SL11*.

Free CRISPR-Cas license to fight hunger

WAGENINGEN UNIVERSITY & Research (WUR) has announced that it will provide potential partners with free licenses to work on its patented CRISPR technology. The license must be applied to gene-editing of plants for non-profit applications. "We hope this contributes to healthier, more sustainable, equitable, affordable and resilient food production for all," says WUR President Professor Louise Fresco during the opening ceremony of the academic year. Worldwide there are over 3,000 CRISPR-Cas related patents, of which WUR holds

several. For five of them (which are jointly owned with Dutch Research Council), WUR decided to provide free licenses. An article in *Nature* was published right after the announcement. "This is really quite unique for CRISPR, in the academic world and beyond. As far as we know, we're among the first to do so regarding CRISPR-technology. We do it, because we simply and firmly believe this is the right thing to do," declares Professor Fresco.

The initiative came from one of the founding fathers of the technology,

Professor John van der Oost. "The potential of CRISPR-Cas cannot be overstated. It is a very versatile technology that could provide new and sustainable ways to feed earth's growing population. We happily share our knowledge to that end, and hope more patent holders will consider doing the same." "The full potential of this technology can only be achieved through long-term partnerships and capacity building," adds professor Fresco. "Together, we could change the way we deal with food security around the world."

I'CONIA Aroma Peach wins FleuroStar Award

DÜMMEN ORANGE'S begonia I'CONIA Aroma Peach received the FleuroStar Award 2021/22. According to the jury, the abundance of double, bi-coloured flowers, the excellent plant habit and the subtle fragrance turn this begonia into an outstanding eye-catcher. I'CONIA was competing against Pelargonium Estelle Appleblossom (Florensis), Petunia Fun House Potpourri (Syngenta Flowers) and Verbena Mr. LavaLava (Selecta one). Begonia I'CONIA Aroma Peach will receive considerable marketing support to turn its go-to-market strategy into a success. The FleuroStar Contest is held annually in the Netherlands and Germany during the Flower Trials week in June. Fleuroselect members can enter their top breeding breakthrough of the season to become the new 'Winner with the Wow Factor'.



Over 30 professionals in breeding, production, trade and retail evaluated the entries on point-of-sale attractiveness and commercial potential

Gene for self-compatibility in potato identified

RESEARCHERS AT THE HYBRID potato breeding company Solynta and Wageningen University & Research have identified, cloned and characterized the gene for self-compatibility in potatoes called Sli (S-locus inhibitor). This discovery will have a profound impact on potato breeding. With Sli defined, breeders can implement hybrid breeding which will allow for faster and focused rather than opportunistic breeding. This focused breeding can quickly bring new resilient and nutritious varieties to the market that will help make potato production become more sustainable. The result of the team's molecular analysis of Sli has been published in the scientific journal *Nature Communications*.

7

Whitefly main vector of tomato leaf curl virus

Bénédicte Lebas, Sven Berendsen, Samer Habash, Smadar Kleiman Shoval, Hubert Lybeert and Rose Souza Richards

8 From its first appearance in 1995 in India, the tomato leaf curl New Delhi virus has spread over Asia, Africa and Europe. The main cause seems to be the tobacco whitefly, which is present all over the world. So far, current scientific information on seed transmission is limited and has not been independently verified. More experiments are required to ascertain whether or not seeds are involved.

Tomato leaf curl New Delhi virus (TOLCNDV) is a plant virus belonging to the genus Begomovirus of the Geminiviridae family (ICTV, <https://talk.ictvonline.org/taxonomy/>). The genus Begomovirus contains more than 280 species, some of which can cause economic losses, such as Tomato yellow leaf curl virus and Tomato yellow mosaic virus in tomato, Pepper leaf curl virus and Chilli leaf curl virus in pepper, and Bean golden yellow mosaic virus in beans. Begomoviruses have either one or two circular DNA

molecule(s); the genome of TOLCNDV is composed of two and has thus been called bipartite genome (EFSA Panel on Plant Health, 2020).



Typical leaf curl symptoms on zucchini

Seed-borne pest versus seed-transmitted pest

According to the International Standard ISPM 38 - International Movement of Seeds - a seed-borne pest is 'a pest carried by seeds externally or internally that may or may not be transmitted to plants growing from these seeds and cause their infestation' (ISPM 38). There are three categories of seed-borne pests, one of them being seed-transmitted pests, which is defined as 'a seed-borne pest that is transmitted via seeds directly to plants growing from these seeds and causes their infestation'. The other two categories of seed-borne pests are: 'Non-seed-transmitted pests that are carried by the seed internally or externally and are transferred to the environment (e.g. water, soil) and then infest a host plant under natural conditions'; and 'Pests carried by the seed, internally or externally, that do not transfer to a host plant under natural conditions.'

Dr. B. Lebas (International Seed Federation, Switzerland), Dr. S. Berendsen (Rijk Zwaan, the Netherlands), Dr. S.S. Habash (BASF, the Netherlands), Dr. S. Kleiman Shoval (Hazera Seeds Ltd, Israel), Dr. H. Lybeert (HM Clause, France) and Dr. R. Souza Richards, International Seed Federation, Nyon, Switzerland, R.SouzaRichards@worldseed.org

Host plants

TOLCNDV can cause severe damage to economically important crops in the Cucurbitaceae and Solanaceae families, including cucumber, melon, pepper, potato, tomato and watermelon. Once tomatoes are infected, a total yield loss can be expected. Pepper plants infected with TOLCNDV show severe and typical leaf curl symptoms. TOLCNDV was initially identified on tomato plants (*Solanum lycopersicum*) showing symptoms of leaf curl in India in 1995. Since the first report, the virus has been reported by different countries across Africa, Asia, and Europe. A study on the natural host range of TOLCNDV, from cultivated and wild species collected in three major cucurbit-producing areas of Spain, found that four wild species across three plant families (i.e. Asteraceae, Cucurbitaceae, Solanaceae) tested positive for the virus. This result suggests that wild plant species surrounding cultivated fields of susceptible plant species can serve as a reservoir of TOLCNDV.

Transmission

TOLCNDV, as all other begomoviruses, is mainly transmitted in the field by its insect vector, the tobacco whitefly, *Bemisia tabaci*. The whitefly is feeding on the phloem of a very wide range of plant species (i.e. it is highly polyphagous) and has a worldwide distribution. The virus remains present in the insect during the lifespan of the insect.

Some strains of the virus have been demonstrated to be mechanically transmissible under experimental conditions using different species of Cucurbitaceae and Solanaceae, including cucumber (*Cucumis sativus*), melon (*Cucumis melo*), ridge gourd (*Luffa acutangula*), tomato (*Solanum lycopersicum*) and zucchini (*Cucurbita pepo*). However, there is no evidence of this mode of transmission in the field. Other strains of TOLCNDV (e.g. TOLCNDV-OM, originally identified from cucumber) are reported not to be mechanically transmissible.

TOLCNDV can be associated with seed. The virus was detected by PCR from surface sterilised of 40 seeds of zucchini (with 10-15 seeds harvested per fruit from



The tobacco whitefly seems to be the main cause of TolCNDV

infected plants grown under natural field conditions in Italy) and on the pericarp, mesocarp, seed coat, endosperm and embryo of naturally infected chayote seed (*Sechium edule*, Cucurbitaceae family).

Under investigation

Potential seed transmission of the virus is still under investigation. Zucchini seedlings (25 seedlings for the two cultivars) grown under experimental conditions from TolCNDV infected seeds tested positive by PCR, indicating that the virus could move through seeds to the germinated plants. However, the experiment did not allow the seedlings to develop into plants for symptoms observation, failing to fulfil the Koch's postulate of demonstrating the causal agent of a disease. Therefore, it is not known whether TolCNDV would cause symptoms from naturally infected zucchini seedlings, which is one of the criteria for a pest to be considered seed-transmitted in the International Standard ISPM 38 - International Movement of Seeds.

Another seed transmission experiment shows that, out of twelve germinated seedlings from 25 naturally infected chayote seed grown under experimental conditions, three plants tested positive by PCR, but none of the twelve plants developed any symptoms. Again, it does not fulfil the definition of a seed transmitted pest of ISPM 38 in which the pest should cause symptoms from naturally infected seedlings. Furthermore, as stated in ISPM 38, 'when the transmission of pests has been observed or confirmed under experimental conditions, it is necessary to confirm that it can also occur under natural conditions.'

There is insufficient scientific evidence that seed to seedling transmission of Tomato leaf curl New Delhi virus occurs in *Cucurbita pepo* or other Cucurbitaceae species. Both seed transmission studies used PCR as detection method. These molecular methods are indirect tests which do not allow the differentiation between viable and non-viable pathogens, and can detect closely related, non-pathogenic organisms that may result in false positive results (ISF, 2013). Furthermore, the seed transmission experiments were conducted on a limited number of seed from very few cultivars. Also, it is not clear whether the germination and growth conditions are representative of the natural field conditions of Cucurbitaceae.

Concluding remarks

The scientific information on the seed transmissibility of TolCNDV is limited; 10-15 seeds harvested from one single fruit of two zucchini cultivars and 25 seeds from chayote. Furthermore, the seed transmission experiments have not been independently verified. Findings from other studies on seed transmission by the same research group on different Begomoviruses - Sweet potato leaf curl virus and Tomato yellow leaf curl virus - were not supported when independently verified. More experiments are required to ascertain, or not, this mode of transmission for this virus. To date, the main mode of transmission of TolCNDV in the field is by its insect vector, *Bemisia tabaci*, capable of feeding on a wide host range of cultivated and wild species. 

Fair prices and easy access to desirable traits

Chris van Winden

10 Almost seven years ago, International Licensing Platform (ILP) Vegetable was launched to improve global and guaranteed access to and use of plant breeding traits for vegetable breeding. The members of ILP Vegetable are very satisfied with the functioning of the association during this time.

ILP Vegetable provides an easy way for vegetable breeders to licence the traits they need at a fair and reasonable cost. The members of ILP will make all of their patents related to vegetable breeding traits accessible to their fellow members under the conditions of ILP.

Transparent

ILP's licensing system is simple and transparent. If a member wants to take a licence to use a fellow member's patented invention, the two parties begin bilateral negotiations. If no agreement is reached within three months, the case goes to arbitration by independent experts. The innovative aspect of the system is the method of arbitration. It uses a 'baseball arbitration' model, whereby both parties submit their licence fee proposal to the independent arbitrators, who then choose the most reasonable proposal. This forces both parties to adopt reasonable positions from the outset, because an unreasonable position will be rejected in favour of a more reasonable competing proposal.

It was expected that the 'baseball arbitration' system would almost always result in parties reaching agreement in their bilateral negotiations. And indeed it turned out that way. So far, there have been no arbitration cases. The question is regularly asked how many licences have been concluded between members of ILP Vegetable. This information is not available because these licences are the results of bilateral negotiations that take place outside the ILP system. Only if these negotiations had not led to a result would the parties' cases end up in the hands of the ILP arbitrators.

Breeder's exemption

In addition to patents pertaining to traits, granted patents on plant varieties as such are also covered by ILP. In practice, this only applies to U.S. patents, because in Europe plant varieties, as such, are excluded from patentability. However, instead of the baseball procedure, a so-called non-assert provision applies, which creates a kind of breeder's exemption under the U.S. patents, provided that the varieties that are bred by using the protected variety, are sufficiently different from that protected variety. This non-assert is free of charge and requires a

Ir. C. van Winden is Managing Director of the International Licensing Platform (ILP), The Hague, the Netherlands, managing.director@ilp-vegetable.org



simple notification to the patentee. Membership is open to all interested parties, regardless of whether they own patents or not. At the start of ILP Vegetable, eleven companies were members of this association. This group of companies comprised both listed companies and family-owned companies. Currently, fifteen companies are members of the association. All major vegetable breeding companies are represented in the association, with the exception of Bayer (Monsanto). These companies are situated in the following countries: China, Denmark, Germany, Japan, France, Spain, the Netherlands and USA.

Sound financial basis

There are currently three small members (less than 100 employees), two medium members (between 100-500 employees) and ten large members (500 or more employees). For these memberships, different levels of annual contribution fees exist. The association has a sound financial basis and it was possible to reduce the annual contribution fees in steps. At the start of ILP Vegetable, the contribution was € 7,500 for small members, € 15,000 for medium members and € 22,500 for large members. This is currently respectively € 4,750, € 9,500 and € 14,250. The number of committed patents amounted to 120 at the start of ILP Vegetable in 2014. By the end of 2020, this number had increased to 250. Committed patents means all published patents and applications thereto owned or controlled – with the right to licence or sublicence to members – by a patentee member and/or its affiliates. These patents are included in a patent register and this register is publicly available via the ILP website.



Members of the International Licensing Platform Vegetable can use 250 patented traits in their breeding programme

environmental risk assessment under EU Directive 2001/18 is considered to fulfil the criteria regarding substantial liability risks or substantial deregulation costs, and is therefore considered to be a 'special trait'. It is stated in the Internal Regulations of ILP Vegetable that, in the event biological material of vegetables with special traits becomes commercially relevant for the global vegetable market and is covered by patents, the members shall negotiate in good faith a comparable solution as agreed under ILP to achieve the object of the association. Discussions are currently underway about new breeding techniques, in particular gene-editing techniques, with the question as to whether or not these techniques lead to GMOS. These discussions are closely monitored within ILP Vegetable, due to a potential impact on the association's regulations. 

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No 'special traits' are included in this patent register. 'Special trait' means a regulated trait which involves substantial liability risks or substantial deregulation costs. Each trait that is subject to an

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Facilitating breeding with patented traits

Darya Chernokova

Access to commercial varieties for breeding purposes and to marketed patented traits will be less burdensome when facilitated by a new 'Agricultural Crop Licensing Platform' (ACLP). This initiative is expected to be launched and operational in 2022.

The ACLP initiative is currently driven by ten European plant breeding companies and trait developers, representing a wide range of agricultural crops. It includes small, medium-sized and large companies. These partners are working jointly on the establishment of a simple legal framework designed to enable access to commercial varieties for breeding purposes and to provide access to marketed patented traits on fair conditions in Europe. Focused on increased transparency, the initiative will eventually include genome edited traits. The ACLP, therefore, intends to be a collaborative source for plant breeding innovations.



Breeders of agricultural crops follow the successful example of their colleagues breeding vegetables

European Patent Convention, Ukraine and Russia). Accessing commercially available varieties for breeding purposes is generally permitted under Plant Variety Protection. If a patented trait is contained in a commercial variety, however, patent laws in most European countries are seen to undermine this access, since they do not consistently provide for a statutory limited breeder's exemption. It is often unclear whether the trait owner would grant a licence to the trait for commercial purposes and, if so, under what conditions. This means that access to specific traits can be a highly complex, time-consuming process, and can come with very diverse conditions which are not predictable by those requesting access.

Quick access

In order to change this, the ACLP will create a clearly defined and transparent setup, in which members can quickly access material for breeding and commercialization through standard licence agreements.

It is envisaged that there will be a membership fee for companies who want to join the platform. Though it is still a little too early in the development of the initiative to be specific about the fee structure, it has been agreed the platform will be a non-profit initiative and, therefore, the membership fee will be exclusively used to finance the platform.

The goal is to facilitate access to traits from innovators in plant breeding, whether public or private, willing to join the initiative, while freedom to negotiate tailored licensing agreements will remain. The use of a standard licence agreement will speed up the conclusion of such licences at low or even zero transaction cost. Financial conditions would remain negotiable between the parties with all other components of the licence standardised. As membership conditions are finalized, the benefits of joining the platform will clearly outweigh the associated costs.

Minimising costs

The ACLP initiative will aim to ensure easy and inexpensive access to licences for all members in general - and for small companies in particular. Special considerations for the requirements and needs of small companies in the model are currently under discussion and aided by some smaller breeders within the group of companies setting-up this initiative.

Small companies will benefit from guaranteed access to and freedom to operate (FTO) for breeding with all varieties commercially available on the open market, including those containing patented traits offered within the platform and maintaining such traits in progenies; minimising transaction costs to obtain a commercial licence; guaranteed FTO for commercialisation of traits contained in newly bred varieties. Finalisation of the framework for the ACLP initiative is advancing, including incorporation of the non-profit legal entity, finalising membership options, technical setup, etc. It is expected to be operational at the beginning of 2022.

Expressions of interest from plant breeding companies in participating in the further development of this exciting initiative are welcome and are obligation-free. Contact info@aclp.eu.

Work in progress to fight late blight

Roger Staats

To this day, *Phytophthora infestans* (literally plant destroyer) continues to pose a major threat to global food production. This year, due to the prolonged cool and wet summer, this fungus-like microorganism has infected potato crops throughout northern Europe, with potentially devastating effects on yields. To sustainably feed a growing world population, to respond to the dramatic changes in our climate, to meet changing consumer demands, and to ensure that we reduce our dependence on chemical crop protection to meet EU2030 Green Deal targets, we need to safeguard and increase potato production. Tremendous resources are being invested in developing resistant potato breeds. Dutch potato breeders are world leaders in developing innovative solutions to combat this destructive pest. In this article, we review the current state-of-the art with a number of specialists in this field.

There's more than one way to ...win a

With 160 members, the NAO represents the interests of 95% of the Dutch potato trade. The members range from cooperatives, traders, exporters, importers, grading stations to companies active in retail packaging. Some companies are active in seed potatoes as well as in potato breeding. In addition to trade members, the NAO has a small number of business members linked to the potato trade. Jan Gottschall, policy specialist at NAO (Dutch Potato Organisation): “The Netherlands is world leader in certified seed potatoes, with a market share of around 60% of the world's certified trade volume. This is mainly due to the major efforts made by our breeders to develop new varieties which will meet the international market and societal needs. I believe our members are amazingly responsive to these needs, for example, the fast-growing demand for more robust or resistant varieties.”

Political aims

“Developing new *Phytophthora*-resistant varieties also matches political aims, like the EU Green Deal, which states that the overall use and risk of chemical plant protection products

should be reduced by 50% by 2030. The same Green Deal also requires 25% of EU's agricultural land to be farmed organically by 2030. Moreover, supermarkets are responding to consumer demands for sustainability; Dutch supermarket chains want to show consumers that their table potatoes are certified under the 'Planet Proof' sustainability scheme.” “To work towards these targets, the Dutch organic potato sector and supermarkets came to an agreement, signing a so-called covenant. The retail sector agreed to stock robust organic potato varieties on their shelves, thus stimulating organic potato growers to use these varieties, as they have genes resistant to *Phytophthora*. But this is also true for conventional potato farmers, as growing resistant varieties can dramatically reduce the use of chemical plant protection products, in some cases by up to 75%.”

Urgent need

“So, there is an urgent need to produce new breeds of potatoes to satisfy the growing demand for sustainability. However, breeding new robust, resistant varieties is currently time-consuming and the ongoing debate



In the 19th century, the pathogen *Phytophthora infestans* caused the 'Great Hunger' in Ireland, leading to a fall in the country's population of 20-25%: 1 million died and 1 million fled the country.

In battle!



Jan Gottschall: 'Growing resistant varieties can dramatically reduce the use of chemical plant protection products, in some cases by up to 75%'

on genetic modification and current EU restrictions on the use of new techniques, like CRISPR-Cas, limit breeders in their race to develop robust varieties to respond more rapidly to the market and societal demand. European organisations are pushing the EU policymakers to liberalise legislation to allow breeders to use genome editing.'

"Luckily, there are more ways to achieve these targets than new breeds and chemical crop protection reduction. A recent innovative WUR (Wageningen University & Research) study looked at how *Phytophthora* infects plants by 'sticking' to the plant and forming a 'sharp knife' to enter the plant. To resolve this, plants can be sprayed with a cheap, eco-friendly product that reduces the leaves' stickiness, making it more difficult for the pathogen to gain entry. With findings like this, bio pest-control initiatives, and the efforts made by breeders, we will make it more difficult for *Phytophthora* to decimate crop production. Hopefully, the combination of breeding, physical solutions and plant protection products will help farmers win their ongoing battle against the 'plant destroyer'."

Organic late-blight blues

Olga Scholten, Programme Leader Plant Breeding, Wageningen UR and Peter Keijzer, Programme coordinator Breeding & Innovative Crops and Cropping, Louis Bolk Institute work together on the Green Breeding programme. One of the projects in this programme is Bioimpuls that is aimed at breeding new resistant potato varieties, especially for the organic sector. The programme, funded by the Dutch Ministry of Agriculture, Nature and Food Quality, has been granted an extension until 2030, as this is a vitally important sector for Dutch agriculture and, therefore, the economy. In 2017, 25 organic potato breeders, producers and supermarket chains signed the covenant.

In the EU Green Deal 2030, ambitious targets were set which affect agriculture as a whole and organic farming specifically (see above). Currently in the Netherlands, organic potato farming is fairly limited in scope and size, although growing. Moreover, organic farmers, in a wet, cool year like 2021, can lose up to 80% of their crop from *Phytophthora* infection, as they use no chemical products, and not all so-called resistant varieties provide full protection: like many pathogens, *Phytophthora* comes in many forms.

Wet conditions

Peter: "In contrast to the last three years, this year's wet conditions are



Olga Scholten: 'Other issues like societal pressure affect what farmers do'

conducive to late blight; just a few wet hours on the leaves and the high humidity is enough for blight spores to germinate. This in contrast to 2019, a very dry year, when we were not even able to infect our own test fields. Wet years can also cause other problems, like hollow hearts or excessive moisture uptake and potatoes explode.

Late blight, if not rapidly treated, can be truly disastrous - within two days, the foliage is killed off, and then it runs off into the soil and infects the tubers, resulting in up to 80% crop loss. So, organic farmers are really suffering this year."

Olga: "Other issues like societal pressure also affect what farmers do. For example, in 2016, public pressure on copper crop-spraying resulted in a covenant to speed up the process of breeding, thereby accelerating the transition to robust potato varieties. It's more than breeding, it's the whole chain, not just developing resistant breeds, they have to be grown and



Peter Keijzer: 'In 2019, a very dry year, we were not even able to infect our own test fields'

get to the supermarket shelves.' Peter: 'The aim was to have 100% robust able-stock varieties by 2020 from organic production; we achieved about 80%, not bad. However, it's worth noting that for half of the season, early varieties on the shelves are not robust, and also that potatoes are grown for chipping and crisping, not just the table.'

"For conventional farmers, wet weather is also a problem, but use of fungicides is still permitted, and *Phytophthora* can be managed chemically. Even here, resistant varieties are becoming more important, as for non-resistant varieties, spraying can be almost weekly in a normal season. In

contrast, with these new races, spraying can be reduced from 15-17 times to 2-3 times to avoid breaking the resistance, as well as the considerable economic and time benefits for the farmers. However, these new varieties also have to have good yield, shape, and taste properties; resilience to *Phytophthora* is but one of the many demands breeders face."

Wild species

Peter: "To develop resistant varieties, breeders use wild species originating from South and Central America; there are an estimated 200-250 original species, depending on who you talk to. The objective for the short-term is to cross existing, advanced genitors developed by Wageningen UR and make the seed available to breeding companies and farmer-breeders. Moreover, breeders cross new resistant genes from wild potato species into modern cultivars, in order to develop new genitors. This takes many years and will lead to useful genitors in the long term, significantly broadening the genetic base for potato breeding. As many as ten different sources of resistance to *Phytophthora* are used in this project. This will enable breeders to combine multiple resistance genes within one new variety. Such 'multiple-gene' resistance is expected to be more durable than 'single-gene' resistance." Researchers from these programmes, working closely together with commercial potato breeding companies and farmer-breeders, are producing new resistant varieties, currently being grown under field conditions. The trend is positive; increasing numbers of new varieties are finding their way to production, both in conventional and in organic farming practice. Peter: "We are leading in this field, and with these projects, we aim to maintain our position. Potato blight and trends in organic farming are global issues; Wageningen UR and the Louis Bolk Institute have the expertise and network to resolve them."

Searching for the breakthrough

'Say potato, Say Agrico': the company's slogan says it all! With more than 75 varieties, Agrico supplies potatoes in all forms, shapes and sizes to world markets. However, to be able to do so, they invest heavily in research. Sjefke Allefs, Research Director at Agrico, looks at the past, present and future of creating new, resistant potato cultivars.

"Strangely enough, when we talk of developing varieties of potato, people immediately think it's something quite recent. Potatoes have been a staple diet in Northern Europe since the 18th century, people discovered that they are easy to grow, nutritious, and have high yields for a relatively small area of farmland. After the Great Hunger in Ireland and Europe in 1845, researchers immediately started looking at ways of growing resistant potatoes to prevent devastating losses caused by late blight – even in pre-Mendelian days. By the early 1900s, a number of resistant sources had been identified, probably from Mexican species like *Solanum demissum* which, amongst other resistant species, could be more easily crossed with our Peruvian-sourced edible potato."

"This first successful resistance gene we now call R1. However, following exchanges between German, UK and US breeders, the Germans noted that their R1-crops were not resistant. This was presumably due to variants of *Phytophthora* which could 'break through' this resistance. So further developments, up to the 1930s, led to R2, R3 and R4 genes from *S. demissum* using relatively rudimentary Mendelian techniques – pre-DNA (1945). The first resistant cultivars were grown in the UK and Germany, however, they too rapidly lost resistance. Breeding for resistance continued up to the 60s, when chemical treatment first became popular."

"After the introduction of chemical treatments, the 'battle was won'; at the time no one considered the negative environmental effects of chemicals; simply *Phytophthora* could be beaten with relatively cheap and effective spraying. As up to then, breeding resistant varieties as a solution had had no real lasting effect, breeding almost ground to a halt. However, breeders started to move in different directions, and moving away from the R factors, in the 1980s-90s, partial, field or quantitative resistance became the path to be trodden in many areas in Europe, as this was seen to be more sustainable."

"Research progressed and, in 2002, the R1 gene was unravelled and researchers also started looking at



Sjefke Allefs: 'There are no 'quick' fixes: the crop wild relatives we take resistant genes from are not viable in Dutch conditions'

how *Phytophthora* worked, identifying hundreds of effectors. So, since then, researchers and field breeders have moved back to looking at the R-genes, as we discovered that the partial resistance was also based on R-genes. Thanks to new techniques, we were able to see that there was a great variation of R-gene expression – in the tuber, in the young leaf, old leaf, etc. So both on the host and pathogen side, we discovered that infection/protection was a much more complex process."

The CBSG programme

"This led to the creation in Wageningen, together with the Dutch potato industry, between 2005-2015, of the CBSG programme – the Centre for Biosystems Genomics. The underlying idea was to screen the complete potato genebank at Wageningen for resistance to *Phytophthora*. All available sources were characterised by their number and genome position. We discovered that the Mexican species had many resistant gene types, never a 'single one', and that there were also, rarer, resistant South American species."

"This was the foundation for most of the current Dutch breeding programmes, and, here at Agrico, we have built on this by developing multi-gene resistant cultivars, which have yet to be commercially grown. We have now developed a selection of resistant cultivars to suit the needs of our different customers, be they 'table', chip or starch potato growers."

"However, the climate this season has once more shown us the need for continuous development. A number of organic growers have contacted us with news of how *Phytophthora* has broken through the resistance of their crop. This wasn't unexpected as, during the research programme, we had also discovered *Phytophthora* isolates which could lead to reinfection. However, the organic growers are seeing absolutely no return on their often-significant investments, and they call us saying that they may turn to other, less disease-prone crops, unless we can come up with more resistant cultivars."

"We call our current resistant breeds the 'next generation', but it's fair to say that, based on the results of the CBSG research programme, we're working on the 'next' next generation, etc., further developing multi-resistant cultivars for future commercial application. We see that a Hungarian bred variety has 4-gene resistance and that, although it's only available on a very small scale, its resistance has yet to be broken, so it's an indication of where we're going. This variety, 'Sarpo Mira', has a mix of resistant genes, strong ones and weak ones."

"There are no 'quick' fixes: the crop wild relatives we take resistant genes from are not viable in Dutch conditions, they are short-day plants, contain high levels of alkaloids, etc., so it takes a lot of breeding and time to produce a commercially viable product."

New directions

"Of course, breeding new varieties is only one of the solutions. Currently there are new, good, non-hazardous fungicides available, so conventional growers can take on resistant varieties which are, for them, commercially attractive, as they result in the need for less spraying, thereby saving time and money: from 12-15 times a season to 3-5 times - significant reductions."

"But other technology can also be of help. In areas like northwest USA, a huge potato-farming area, the conditions are cool, but very dry: all the crops are irrigated, and this doesn't give *Phytophthora* a chance, as the plant's leaves are never continuously wet. We also now have accurate meteorological apps and that can lead to better spray planning. There are even systems that suck the fungal spores out of the air. Drones can soon be used for field-scouting and they can accurately identify the first symptomatic plants. They can be programmed to fly all day and report the coordinates of the symptomatic plants, and then the farmer can spray on time, preventing losses. However, I have to say that, in the Netherlands, automated drone flying (without an operator) is not permitted (yet)."

"However, even with all these other developments, I'm extremely optimistic that we'll find the right multi-gene resistant varieties, which will be especially of value to the organic farmers, as they, more than others, are fully dependent on our new 'next generation'." 



Comeback of a Transgene Garden Plant

Orange petunia finally returns

Monique Krinkels

18 Four years ago, petunia breeders, producers, sellers and consumers were in shock: their beloved orange petunias appeared to contain a maize gene. About hundred transgenic petunia varieties in cultivation and in market circulation had to be destroyed. Breeders of the German company Westhoff took it upon themselves to apply for a non-regulation status in Canada and the USA.

In May 2017, the family-owned garden plant breeding and producing company Westhoff received the mind-blowing news that their beautiful orange petunias were a transgression of the European laws. "Without being aware of it, we had introduced 18 transgenic petunia varieties onto the market. Twelve of which had either an orange colour or were bicolour combining orange with another hue," explains Dr. Diro Terefe Ayana. Somewhere in the past a transgenic petunia had unintentionally entered the collection of parental lines. Westhoff is Germany's largest producer of ornamentals. From its head office in Südlohn, in northwest Germany, the company ships millions of garden plants annually to retailers and mail order companies across Europe. Licensed companies around the globe serve the consumers elsewhere. The vibrantly orange petunias were among the most popular garden plants.

Ripples in the pond

The problems all started in 2015, when Finnish biologist Professor Teemu Teeri walked by a planter at Helsinki Central Railway Station. The orange petunias reminded him of an experiment he had read about some thirty years before. He reported his encounter at the Finnish Food Safety Authority, Evira, who had the plants analysed. They concluded that the petunias were the variety 'African Sunset' bred by Takii and that they contained the A1DFR-gene from *Zea mays*. Besides the maize gene, they also had the antibiotic resistance gene *nptII*.

The origin of the transgenic petunias was the mutant RLO1 created by Peter Meyer and colleagues at the Max Planck Institute in Cologne, Germany, and described on 17 December 1987. The A1DFR-gene had caused a new pigmentation pathway, creating a salmon-coloured flower. Their scientific discovery attracted the attention of the breeders of Sluis & Groot (today Syngenta) who started to breed a more vivid, orange-coloured petunia, which they obtained by 1995.

At that point, the search for orange ended, as opposition against transgenic plants increased. Both the Max Planck Institute and S&G destroyed all their transgenic petunia plants and seeds. No one knows how the transgenic petunia became part of the breed-

ing programme of several companies, but the fact is that it did. And once a plant is part of a breeding programme, no one will scrutinise its ancestry as breeders' exemption allows it to be used.

Saved from destruction

In 2017, the EU authorities ordered all petunias containing the maize-gene, in cultivation as well as in market circulation, to be destroyed, and a directive by the US and Canadian agencies followed suit. "Consequently, Westhoff destroyed all of the transgenic petunias from our greenhouse," explains Dr. Diro Terefe Ayana. "It was not allowed to keep transgenic petunia seed under conventional breeding conditions. Molecular biology laboratories, having permission to research on genetically modified organisms, may store the seed for inspected research purposes. Fortunately, we maintain our collection of varieties as an in-vitro stock in a collaborating laboratory. So, we secured some of those unintentional transgenic varieties as an in-vitro plant in Canada."

By the end of 2017, Westhoff applied for a deregulation of all petunia varieties carrying the A1DFR-gene in Canada and the USA. It was expected that it would not be too difficult, as the construct is well known in the USA. The maize-gene has been comprehensively tested and was declared safe for the environment and human consumption by the Environmental Protection Agency (EPA), as well as the Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA). Dr. Manfred Mehring-Lemper, at the time head of the breeding department, and his successor Dr. Diro Terefe Ayana, were the driving force behind this multi-year effort.

"Westhoff's orange petunia varieties have distinct, brilliant colours and there was strong demand for them in the market, prior to being removed. Many customers asked us whether we saw any possibility of reintroducing the varieties. Certainly, economic interests also played a role for the entire market. We think these genetics are worth making available again. It was also clear that, using classical breeding methods, no comparable varieties are expected in the foreseeable future."

Time-consuming effort

"The process from the first contact to the final deci-



Dr. Manfred Mehring-Lemper (left) and Dr. Diro Terefe Ayana were the initiators in applying for deregulation of the maize-gene containing petunia

sion took three years and four months. The application was based on literature review of the A1DFR transgene in petunia in general and a DNA sequence-based verification of Westhoff's A1DFR-petunia. It was a huge effort and took months to complete the documents. The comments we received from the USDA authorities were very helpful. The document was revised several times and resubmitted, but the USDA was very supportive throughout the process." Canada was the first country to allow the continued sale of the orange petunia and the US quickly followed. Would you consider applying for approval in other countries? Australia? Europe? "No, not this time. We want the market to dictate which plants Westhoff introduces, so if it is appropriate and we have demand from the growers and home gardeners of a country, then it is definitely something we would consider."

Sharing the burden

The approval marks a milestone, not only for Westhoff, but for the entire industry. The change from regulated to non-regulation status allows for all A1DFR petunias to be sold free of restrictions, regardless of the company that developed the variety. "This approach is consistent with Westhoff's long-held corporate mandate to ensure that novel breeding is brought to the marketplace, without restrictions, to allow for a competitive, innovative and robust floriculture industry."

It sounds logical that this application would have been a shared effort by all petunia breeders. "Informally, we discussed with many petunia breeding companies the possibility of preparing a petition for deregulation in the USA. At that time, many of them assumed the petition preparation would be too

costly, rigorous and unrealizable. Regardless of all the doubts, Westhoff proceeded with the application solely. Although many of the companies declined to collaborate with Westhoff on this project, the final decision to deregulate helps all breeders that are interested in developing petunias containing the A1DFR gene, which they have started to do on their own timeline."

Classical breeding

"We are following classical breeding techniques to obtain orange petunia as well, but the unintentionally introduced with well-known brand names are very famous and have received more attention. Up to now, the petunias with the A1DFR gene are showing an extra brilliant colour, compared to the ones bred by classical breeding.

"Keep in mind that Westhoff has only ever used classical breeding to develop these A1DFR gene containing plants. The original modification happened unknowingly decades earlier, but the gene has been carried forward via classical breeding procedures," according to Dr. Ayana. "Westhoff is not actively creating new transgenic varieties using gene insertion methods, nor do we have any interest in doing so." From now on, American gardens can be brightened up with orange petunias. "We currently have fourteen A1DFR-petunias entering the US market, but again, with the status of the plants changing, any breeder can begin to develop and introduce varieties that contain the gene." Among the transgene Westhoff varieties are 'Mandarin' (see cover), but also 'Hell's Fury' and 'Hell's Bells'. We will have to wait and see which of the 100 or so A1DFR gene-containing varieties that were banned in 2017 might return to the market. We will look for them in Spring 2022. 

Recalcitrant crops budge into new protocol

Wessel Holtman and Bert van Duijn

20 Among many other crops, tomato has been known as a highly recalcitrant crop in doubled haploid technology. For a long time, it seemed impossible to obtain doubled haploid plants via gametes. Fytagoras has succeeded in developing a method also for this difficult crop that has already been applied successfully for several customers.

In the breeding strategies and programmes of many crops, genetically pure homozygous lines are highly desired. In pure homozygous individuals, the chromosome pairs (one originating from the father and one originating from the mother) are identical, i.e. the alleles on both chromosomes are identical. An allele is one of two, or more, versions of the same gene at the same place on a chromosome.

These homozygous lines make qualitative and quantitative phenotypic selection more efficient, as no hidden properties are present (all genes are present in just one version, in contrast to heterozygous individuals). In addition, these lines form the basis for F1 hybrid seed production, i.e. a crossing between two homozygous parents resulting in identical offspring. In homozygous parent lines, lethal and weak alleles are eliminated as they cannot survive since no strong counter allele is present.

Finally, pure homozygous lines are desired for establishing chromosome maps, whole genome sequencing, bulked segregant analysis (BSA), which is used for detecting markers associated with traits in segregation populations and for mapping of quantitative trait loci (QTLs). There are different methods in plant breeding to obtain (pure) homozygous lines. For different crops, different methods may be applied. In this article, the different methods will be briefly discussed and, subsequently, the focus will be on the production of doubled haploids (DH) via androgenesis.

Methods

Generally, there are three ways to obtain (pure) homozygous plants, more or less applicable to different plant species: back crossing breeding, doubled haploid technology and the haploid inducer system. Back crossing is a relatively simple way to obtain nearly homozygous lines in breeding programmes. These back crossings (inbreeding) result in a more homozygous offspring every cycle, however, complete (100%) homozygosity is never achieved. To obtain an applicable level of homozygosity, up to ten back crossing cycles are required. This is labour intensive, as well as time-consuming (depending on the crop, these back crossings may take up to ten years). Methods like single seed descent (SSD) may be used to speed up the propagation and selection

process, but still several years are needed to obtain stable lines.

Hence, the need for methods that deliver 100% homozygous lines in a faster way. The solution to this is the development of doubled haploid (DH) lines via DH technology.

Doubled haploids

DH lines are produced in tissue culture from haploid cells (containing only one copy of the genome) that are forced to divide, duplicate their chromosomes (become doubled haploid = homozygous diploid) and form embryos. The embryos can germinate and form new DH plants. In this respect, DHs are a revolutionary achievement in plant breeding, because completely homozygous plants can be produced within one generation. DH production can be achieved through androgenesis, gynogenesis or parthenogenesis, depending on the species. A chromosome-doubling step is mandatory when spontaneous DHs are not regenerated. This can be achieved by using antimitotic compounds to double the ploidy level of haploid plants.

To obtain DH lines, gametes from meiotic cells are harvested and, from them, plantlets are generated (androgenesis or gynogenesis). Alternatively, haploid embryos can be produced by 'pollination' with pollen (parthenogenesis).

The haploid cell can be either a microspore from an anther or an ovule from an ovary, depending on the species. The regeneration of plants from microspores or ovaries is a one- or two-step process. If the protocol directly induces embryo formation, a plant can be produced in one step. In many cases, first a callus (non-differentiated tissue) is produced and, in a second step, embryos and plants. Gametic cells from meiosis can also be developed into haploid embryos, via parthenogenesis. Thus, the process of DH production always involves a gametic haploid step from which haploid or DH plantlets will be regenerated. The in vitro culture of gametic cells in androgenesis and gynogenesis techniques requires the original gametophytic pathway of the gamete to be redirected towards a sporophytic pathway, where plantlets can be regenerated. In this technique, androgenesis is the most common method to produce DHs, although, for a number of species, gynogenesis is preferred. In

Part 1

Development of an initial protocol

Phase 1

Formation of multicellular structures

1 Starting material

GO / NO GO

2 Induction of cell division

GO / NO GO

3 Formation multicellular structures

GO / NO GO

Phase 2

Embryo and plant formation

GO / NO GO

Part 2

Optimization of the protocol

Application on other varieties

Production of diploid plants

Overview of Fytagogoras' approach for doubled haploid plant production

Tomato DH plants in the green house for seed production



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androgenesis, isolated microspores or anthers containing microspores are cultured in specific induction media to induce the formation of embryos or callus. The ploidy level of the regenerated plants can differ depending on the cell events related to spontaneous or induced chromosome doubling. Haploids, doubled haploids, mixoploids and tetraploids can be produced during the in vitro DH process.

Potential pitfalls are that some protocols are prone to also produce somatic embryos from anther or ovary tissues. These plantlets are diploid but have an identical genomic background to the mother plants from which DHs are expected to be generated. Also, clones of one individual DH may arise, especially when a callus step is involved in the production of plant material. In parthenogenesis methods, the formation of an embryo from an egg cell without fertilization takes place. Irradiated pollen is used to induce egg cells to form haploid embryos. These embryos only inherit the maternal set of chromosomes. Such embryos

germinate in vitro and develop mostly haploid plants, but sometimes also mixoploid or spontaneously chromosome doubled haploid plants.

Haploid inducer

Finally, the third method to obtain DH plant is the use of haploid inducer (HI) lines. Pollen or ovules from paternal or maternal HI lines allow for fertilization and embryo development but only one set (maternal or paternal) will survive in the process. Hence, haploid embryo's (plantlets) are produced that can be treated with antimitotic chemical to achieve chromosome doubling, resulting in a (pure homozygous) doubled haploid. For example, maize spontaneous developed HI lines are available, while for some other species HI lines are made through genetic modification, e.g. with CRISPR-cas techniques.

Fytagogoras approach

Amongst different methods described above,



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Tomato DH plants in tissue culture obtained via androgenesis from a single parent line. The high diversity in the phenotype of the different DH plants showing the genetic potential is remarkable

Fytagoras has focused on androgenesis for production of doubled haploids. For this, haploid cells from gametes are collected and provoked for dividing and for doubling of their chromosome store in order to obtain a stable doubled haploid line within only one generation. In contrast, with traditional methods, it may take 6 to 10 generations of self-fertilization to obtain a variety that has fixed major characteristics (which can nonetheless be unstable).

Fytagoras is a research company, which over the last years has become a specialist in doubled haploid technology. As a service to customers, Fytagoras offers DH protocol development as well as production of doubled haploids from existing customer-owned lines on demand. A major milestone has been the realization of protocol for production of tomato doubled haploids in 2017. Tomatoes have been known for many years as a 'very recalcitrant crop' and it seemed impossible to obtain doubled haploid plants through gametes

Obviously, an in vitro cultivation stage is necessary in order to regenerate a complete plant, as the rate of natural mutation from haploid (sterile) to diploid (fertile) is only 1 to 10%, depending on the variety. By careful treatment of isolated reproductive cells and the application of different stress and growth conditions, these cells can double their genetic material and can be stimulated to divide and form multicellular structures, embryos and plants. Typically, for some species, agents are needed to enhance the conversion from haploid to diploid cells and make it useful for practical application. For all species, and often also for every variety, optimal conditions for the generation of doubled haploid must be selected and implemented.

Phasing approach

The strong point from the Fytagoras' approach is the phasing approach (see scheme) combined with practical experience in donor plant selection and treatment selection. Selection and growth of donor

plants is the key to success, providing flower material for isolation of viable gametes, which are used for tissue culture activities. Fytagoras, a company active in research projects involving horticulture and seed technology, has implemented a three-step programme. In the first phase, the quality, vitality and responsiveness of the cells are determined. In the following phase, a basic protocol development is carried out, while in the third phase, the protocol is optimized for efficiency.

Crops

Protocols for production of doubled haploids plants have been published for many species already and are commonly used in breeding programmes worldwide. Typically, doubled haploids are produced for open field crops like barley, maize, wheat, rice, tobacco and rape seed, but also for vegetable crops such as cucumber, carrot, pepper, eggplant, tomato and many other crops. Also, with increasing business and specialization over the last years, more breeders of ornamentals make use of doubled haploid technology. However, not all the species respond well enough to doubled haploid technology and even within species, tremendous variation may exist in responsiveness of different varieties for protocols.

Many species are still considered as recalcitrant to these treatments, including many of the most important crops worldwide, such as cotton and coffee. Despite the work of many groups, little is still known about how to overcome recalcitrancy. Initiatives have been launched about identification of genes, and other factors which are related to responsiveness of genotypes for doubling of haploid cells and induction of cell division. Clearly, a systemic approach is needed, strong dedication and an organization which ensures the continuity of the research programme. Commercial agreements with seed companies may be a solid base, ensuring progress over the years and dissemination of the results. At least for tomato, this approach turned out to be successful. 

Study reveals Caucasian descent

Monique Krinkels

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Cultivated lettuce (*Lactuca sativa*) is believed to be domesticated from *L. serriola*. Until recently, its origins and domestication history remained obscure. Researchers at Wageningen University & Research (WUR) sequenced a total of 445 *Lactuca* accessions, including major lettuce crop types and wild relative species, and generated a comprehensive map of lettuce genome variations.

Technical details about this research can be found in: Whole-genome resequencing of 445 *Lactuca* accessions reveal the domestication history of cultivated lettuce, by Tong Wei, Rob van Treuren, Xinjiang Liu, Zhaowu Zhang, Jiongjiong Chen, Yang Liu, Shanshan Dong, Peinan Sun, Ting Yang, Tianming Lan, Xiaogang Wang, Zhouquan Xiong, Yaqiong Liu, Jinpu Wei, Haorong Lu, Shengping Han, Jason C. Chen, Xuemei Ni, Jian Wang, Huanming Yang, Xun Xu, Hanhui Kuang, Theo van Hintum, Xin Liu and Huan Liu, in *Nature Genetics* 53 (2021), pages 752–760, <https://www.nature.com/articles/s41588-021-00831-o>.

Lettuce (*Lactuca sativa L.*) is an important vegetable crop in the Compositae (also known as Asteraceae) family and is widely consumed as salad greens in many countries. Where lettuce originated has, however, remained unclear. Lettuce was first depicted on wall paintings of Egyptian tombs around 2,500 BC, making it one of the oldest known vegetable crops. At the time, the plant had a clear stem on which thorny leaves grew and it produced a milky coloured, bitter tasting juice. It was widely known for its aphrodisiac and medical uses. According to a text on the Horus Temple in Edfu, Egypt, it was the main offering to Min, the god of fertility.

According to a clay tablet that listed his plants, lettuce was cultivated in the garden of the Chaldean king Marduk-apla-iddina II in Babylon in 700 BC. At the time, it had already become an appreciated vegetable as it was grown along with onions, garlic, leeks, cucumbers, turnips, as well as herbs and the so far unidentified 'slave girl-buttock' in the royal kitchen garden. Later, the Greeks and Romans started breeding lettuce to create a more appetising vegetable.

Several hypotheses were proposed regarding the domestication centre of lettuce, including Egypt, the Mediterranean area, the Middle East and Southwest Asia. Rob van Treuren and Theo van Hintum from the Centre for Genetic Resources, together with researchers from BGI Genomics in Shenzhen, China, traced back the family tree.

Centre for Genetic Resources

The Centre for Genetic Resources in the Netherlands (CGN) is a Dutch gene bank. It manages a collection of 2,500 lettuce varieties - the largest, most complete and best-documented lettuce collection in the world. "About 1,500 varieties in the collection were once cultivated by farmers somewhere in the world, and a further 1,000 approximately are wild lettuce plants from roadsides and nature reserves," explains Rob van Treuren. "In collaboration with BGI, the DNA sequence of all 2,500 species will be determined, including an analysis of genetic variants and the differences and similarities between these variants. The results of the first 445 lettuce varieties have led to a publication in *Nature Genetics* about the origin and breeding history of the crop."

In-depth analyses of the population structure and



Babylonian scribes carefully listed the plants in the royal garden of king Marduk-apla-iddina, the biblical Merodach-Baladan, 721-710 BC and 703 BC on a clay tablet with cuneiform inscriptions (Photograph: The Trustees of the British Museum)

demography revealed that lettuce was first domesticated near the Caucasus, which was marked by loss of seed shattering. "We also identified the genetic architecture of other domestication traits and wild introgressions in major resistance clusters in the lettuce genome. This study provides valuable genomic resources for crop breeding and sheds light on the domestication history of cultivated lettuce."

Wild relatives

Wild relative species are often used in crop breeding as a source of novel traits, such as growth vigour and disease resistance. The *Lactuca* genus consists of about a hundred species, of which approximately twenty belong to the lettuce gene pool. The primary gene pool (GP1) is composed of completely infertile taxa formed by *L. sativa* and the wild species *L. aculeata*, *L. altaica*, *L. azerbaijanica*, *L. dregeana*, *L. georgica*, *L. scarioloides* and *L. serriola*. The secondary gene pool (GP2) is formed by *L. saligna* alone, while the tertiary gene pool (GP3) includes *L. virosa* and another ten *Lactuca* species. *L. serriola*, *L. saligna* and *L. virosa* are the main species extensively used in breeding and compose the majority of wild *Lactuca* germplasms stored in genebanks worldwide.

"Advances in DNA sequencing technology make it feasible to study the genetic architecture in such germplasm collections. A previous RNA sequencing (RNA-seq) study of 240 lettuce accessions demonstrated that different crop types of cultivated lettuce were derived from a single domestication event." However, the domestication history of cultivated lettuce and the genetic basis of human selection remained largely unknown.

"In this study, we sequenced 445 *Lactuca* accessions



The compass plant (*Lactuca serriola*) is the closest wild relative of cultivated lettuce. The common name refers to its upper leaves that twist round in the sun to hold their margins upright

from 47 countries, comprising the major lettuce crop types and wild relative species. More than 208 million sequence variants were identified, from which we revealed the population structure of the genebank collection and the domestication history of cultivated lettuce. In lettuce breeding, the species of the primary gene pool are used widely as there is no reproductive barrier within the group.”

“A comprehensive variation map, including 179 million single-nucleotide polymorphisms (SNPs), 30 million insertions/deletions (indels) and 244,866 structural variants, was constructed, from which we analysed the phylogenetic relationship within the gene pool species and the domestication history of cultivated lettuce. The genetic architecture of domestication traits and introgression regions in resistance clusters were also identified. These sequencing results provide a valuable resource for lettuce research and breeding in the future.”

Gene flow

The Middle East, which in a broad sense includes Transcaucasia, Iran and Asia Minor, represented a major domestication centre for important crop species, such as wheat, barley, oat, chickpea and lentil. By analyzing wild germplasms collected from broad habitats across the Eurasian continent, we hypothesize that the Caucasus was probably (or close to) the centre of lettuce domestication. The domestication time of cultivated lettuce was estimated to be around 4,000 BC, predating the records of lettuce paintings

on the walls of Egyptian tombs and temples. Another interesting result is the gene flow from Southern and Western European *L. serriola* populations to cultivated lettuce, which agrees with an early cultivation history in Greece and Rome, and later, the European mainland. These demographical results were further elaborated by the phylogeny within the genomic regions associated with domestication traits in cultivated lettuce.

The genetic determinant region of seed shattering in cultivated lettuce shared a close relationship with the Caucasian *L. serriola* population, which marked lettuce domestication as a seed crop in this region. The population from this area showed the highest nucleotide diversity. In contrast, the entire leaf morphology, which was determined by a 600-kb region on chromosome 3, was shared between cultivated lettuce and a group of entire leaf Southern European *L. serriola* accessions.

L. aculeata represents another potentially important gene pool, as its phylogenetic position, distinct from other GPI species, suggests a different genetic repertoire. It is plausible that the wild progenitor of cultivated lettuce was first domesticated as a seed crop near the Caucasus, and leaf lettuce was later produced in Southern Europe with an introgression from the local wild population.

Important collection

“Overall, our study constructed phylogenetic relationships within lettuce gene pool species and revealed the genetic basis of human selection during lettuce domestication. The genome sequences and the variation map generated in this study will serve as a valuable resource for lettuce research and breeding in the future.” According to Rob van Treuren and Theo van Hintum, the study perfectly demonstrates how much information can be extracted from DNA data from a gene bank collection. It also shows the importance of preserving and protecting biodiversity and genetic resources for a sustainable food supply in times of climate change and a growing world population.

“Our study provides new insights regarding accession identity and genetic resources for crop improvement, demonstrating the value of whole-genome sequencing in the management of crop collections and the utilization thereof.”

Size matters!

Roger Staats

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New varieties, new colours, new sizes: decorating people's homes and offices with flowering plants is big business. But recently - although big is still beautiful - a new range of small, seductive flowering plants are filling retail shelves and consumers' offices and homes.

Why does size matter?

“Imagine the following scenario. You're visiting a friend and decide to take a small gift - a flowering plant. But a 12 cm pot plant is quite a large-sized gift and to make it perfect, you need a pot - a large pot! Altogether a lovely but expensive, and possibly a bit ‘over the top’, gift. So, what if there was an equally lovely, but smaller, orchid in a 7 cm pot for sale, wouldn't that be better?” says Robert Kuijff, product manager orchids at Anthura, in the Netherlands.

Business acumen

Anthura specialises in orchids and Anthurium. The company has been around for more than 80 years. “The company has seen strong growth, basically thanks to good business acumen - foresight, innovation and making sure that it grows what consumers want and need. Predicting new trends. In 1995, we also took over a struggling German breeder, and this included the orchid division. That is how the genus orchids became our diversification. The takeover brought in many years of orchid breeding experience. That's how we got started - and now we're world leader - in orchids. And that in 25 years.

“This success is due to our people's breeding skills, entrepreneurship and, of course, our view of the market. Commercial dealers and consumers were and are used to selling and buying plants in 12 cm pots, with plants around 60 cm high. With Dutch homes having large windows, these are the ideal plants for front window dressing, as any drive around Dutch suburbs will show you. All is well, you may think, but markets are never static, consumers have changing lifestyles, changing desires, and we have to respond to that.

“That's what's happening now. Homes are getting smaller, new houses are being built with smaller windows, people have a workplace in their homes, many rapidly changing trends, but one thing remains true: people do like livening up their homes with flowering plants. Why not add a bit of colour to your desk with a small Anthurium or orchid, one in the smallest room, and one or two around the kitchen?”

Small is the new big

“However, it's one thing to know this now, but developing new (smaller) sizes of plants can take as much as ten years, with the associated development budgets, slow growing and chances of failure. That

means we have to be ahead of the consumer and know what's going to happen. We observe and anticipate, sometimes a risky business. Just as challenging as trying to convince the growers and retailers that the new trend will be a viable, commercial success.

“How do you ‘grow’ smaller plants? How simple is that? If I took you through our demonstration facility in Bleiswijk, I'd tell you that, in Europe, 150 million orchids are grown every year for the European market - that's one for every four of the European population. This was the standard 12 cm pot, 60 cm high. We then developed a 15 cm pot for larger houses and offices. But our breeders said, we also have homes with kitchen windows, smaller spaces, so we started developing a 9 cm pot - with orchids of 40 cm high. “The growers were cautious: ‘a small plant has a small margin.’ But there's ‘gift’ added value - i.e. orchids in a small glass cylinder, and importantly production costs are slightly lower, and smaller plants are easier and cheaper to transport. Luckily, one of the grower's wives said: “that's exactly what people want”, convincing her husband. They started first, and are now extremely successful in 9 cm plants, demonstrating that the consumer market was ready for this small size. We did the same for Anthuriums and they are now extremely popular.”

New Year's gift

“We have to propagate plants - it's all tissue culture, so we need many hands for production. We propagate plants in our own facilities in China and North Macedonia, as well as in the Netherlands. China is the largest world market for pot Anthuriums. We have our own facility there, with our own lab. We're now well known in China, we were one of the first to set up a company there, mainly for Anthurium. However, orchids came mainly from Taiwan, where they are the national emblem.

“At the time, the Chinese bought two colours of orchid, purple and white, with large flowers and cascading flowers. The orchid is a traditional New Year's gift. To compete at an exhibition in China, I took three colours of orchids with me: red, purple and white. I also took some small flowers with me and the Chinese picked up on that. They wanted the small ones as many Chinese live in small homes. We are now selling small-sized plants there. We offer



A small Anthurium or orchid will brighten up a desk or kitchen window

different colours, as we see the consumers changing their appetite for different sizes and colours. I believe that we have a huge future for small sizes in China. The world market for orchids is estimated at about 350 million plants, annually. We estimated a market of 20 million for 6 cm plants ten years ago. Based on an example from a Danish grower, we started developing a 6 cm pot orchid for the gift market. It has taken us ten years to build a good range of flowers in this size of pot.”

Sustainable strategies

“We were the first company in the world to chart the Anthurium and orchid genome - together with KeyGene. We still breed smaller sizes using classical breeding techniques, as we don’t use any gene technology. We are now getting to the point that we can read the genome, and hope in the future to use new techniques like CrisprCas to develop new varieties more quickly.

“We have developed many new varieties for different target groups and diverse home situations. For example, 12 cm pots with low bushy flowers less than 40 cm high, can be put on a coffee table without obscuring the tv. We have developed new pastel-shades of flowers, but these were not popular at first.

The growers believed that ‘soft’ colours would not sell. Here again the customers proved them wrong. We have to really watch the market and then take risks to develop new varieties and convince our growers to take a leap of faith and produce them.

“We ensure that we operate as effectively and sustainably as possible. We have little waste, although breeding and growing orchids requires a warm environment - 28°C during the vegetative period. Therefore, our facilities use geothermal warmth and solar panels for energy. Our specialists also look at what the growers needs - for example, using less chemicals. It’s more than developing new colours and sizes. Our new research station is at the forefront of these breeding developments, and because of our focus on two types of plants, we are able to work more effectively and efficiently than breeders with a large and diverse product range. We work with very hygienic conditions, as our process starts in the laboratory. As members of the FSI 2025 - the Floriculture Sustainable Initiative - we are ‘clean producers’ and are committed to keeping our environment as ‘clean’ as we can.

“So, to come back to what do we do best: we find out what the consumer wants and take the risk. That’s our business model and it’s serving us well!” 

SOWING A VIBRANT FUTURE



The International Seed Federation, Asociación Nacional de Obtentores Vegetales (ANOVE) and Asociación de Empresas Productoras de Semillas Selectas (APROSE) warmly invite you to the ISF World Seed Congress 2022 in Barcelona, Spain from 16-18 May. Barcelona, the cosmopolitan capital of the Spanish region of Catalonia, is a melting-pot of cultures and famous for its art and architecture. Enjoy its dynamic spirit while living the ISF World Seed Congress experience.

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Joyce Woudenberg, Hubert Lybeert, Smadar Kleiman-Shoval and Rose Souza-Richards

To facilitate the international movement of seed, ISHI-Veg has developed and validated a new confirmation method for detecting *A. citrulli* in melon seed. This grow-out in sweat boxes is faster, less expensive and uses less space, while giving fewer climate variation risks because of the fixed environment used in the growth chamber.

Qualitative results of the inter-laboratory comparative test.

Dr. J.H.C. Woudenberg, Dr. H. Lybeert, Dr. S. Kleiman-Shoval and Dr. R. Souza-Richards are members of the International Seed Health Initiative for Vegetable Crops (ISHI-Veg), International Seed Federation, Nyon, Switzerland, R.SouzaRichards@worldseed.org

• **Acidovorax citrulli**, formerly known as *A. avenae* subsp. *Citrulli* is a Gram-negative, obligately aerobic, biotrophic bacterium that causes seedling blight and bacterial fruit blotch of melon and watermelon. Bacterial fruit blotch of melon and watermelon is a sporadic disease but, in a favourable environment, it becomes devastating and may cause 100% loss of marketable fruit. Infected seeds are one of the primary sources of inoculum in commercial fruit production field. The recommended management strategy is to evaluate seed productions by field inspection or seed testing of a representative sample of each seed lot.

Traditional tests

The preferred method to detect *A. citrulli* is a seedling grow-out test conducted under favourable conditions for symptom development. Seeds are planted in a sterile potting mix in a greenhouse free from other sources of *A. citrulli*, usually a greenhouse dedicated to seed testing. Relative humidity in the greenhouse is maintained above 55% and temperature is main-

and is standardised by the USA National Seed Health System (NSHS) can be accessed at the website www.seedhealth.org.

For higher throughput, a seed extract qPCR assay was developed by the International Seed Health Initiative for Vegetable Crops (ISHI-Veg) as 'pre-screen' in 2017. PCR is considered an 'indirect' test and is used as pre-screen, meaning that a negative test result indicates that the tested seed lot is free from the bacteria, whereas a positive PCR result must be confirmed by a direct test – grow-out assay – to confirm viability and pathogenicity of the bacteria.

Grow-out in sweat boxes

In 2016, ISHI-Veg initiated a project with the objective of developing and validating an internationally accepted method for the detection of Acidovorax citrulli on melon seeds, using a grow-out test in sweat boxes and confirmation of symptoms by a biological assay. The advantage of the sweat box grow-out method over the greenhouse grow-out is that it is faster, takes less space and, as it is performed in a climate chamber, the environmental conditions are easier to control. It could be an alternative to the validated greenhouse grow-out method and an option to confirm a positive result obtained after the seed extract q-PCR (SE-qPCR) pre-screening assay.

In the ISHI-Veg International Technical Group Cucurbits a sweat box grow-out method was developed, based on protocols which were already in use in individual ISHI-Veg member companies. Validation of this sweat box grow-out method was conducted according to the ISHI-Veg guidelines for the validation of seed health tests. Validation is a requirement of method development, and it is a process that determined the suitability of a method for its intended purpose. ISHI-Veg has identified six performance criteria to make the assessment, which are in line with those used by other accreditation bodies for developing seed health methods: analytical specificity, analytical sensitivity, selectivity, repeatability, reproducibility and diagnostic performance.

Analytical specificity

Before the sweat box grow-out assay can be used in routine testing, it is essential to verify that it gives reliable and consistent results when detecting *A.*

Lab nr	Number of samples (Nº positive/Nº tested)		
	Healthy	Medium contaminated	Highly contaminated
01	0+/13	8+/13	12+/13
02	0+/13	10+/13	13+/13
03	0+/13	12+/13	13+/13
04	0+/13	11+/12	13+/13
05	0+/13	8+/11	13+/13
07	0+/13	2+/9	11+/12
08	0+/13	3+/13	13+/13

tained between 24-38°C. After 16-21 days, each seedling is carefully inspected for symptoms. Isolations are made from seedlings showing any symptoms of disease and *A. citrulli* is identified using biochemical, DNA and biological assay. This method takes 30-35 days to complete.

Elaborate precautions must be taken to ensure that cross-contamination of seedlings does not occur in test procedures. A detailed step-by-step seedling grow-out method that describes such precautions

“Making vegetables available for everyone.”

This is the ambition of Hilal Kanik and Canan Acarbulut, tomato breeder and selection co-ordinator tomato respectively, both working for Rijk Zwaan in Antalya. Read their story on rijkzwaan.com.



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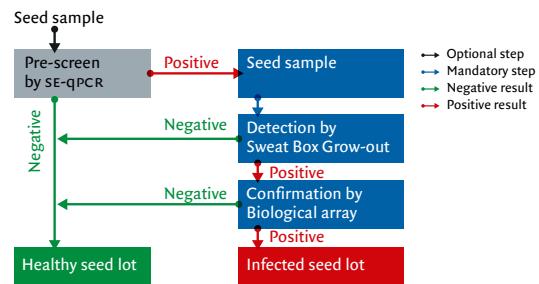
When *A. citrulli* lands on healthy leaves, it migrates through open stomata into the sub-stomatal intercellular spaces where it multiplies and induces water-soaked lesions

A. citrulli. The assay should give a positive reaction with the target pathogen but not with, for example, closely related non-pathogenic organisms, this is called the analytical specificity. The analytical specificity of the ISHI-Veg sweat box grow-out assay was evaluated during a project followed by the European EU-PHRESCO network (Consensus Detection and Identification Protocol for Acidovorax citrulli in cucurbit seeds, project started in 2016, [DIP-ACIT]). Eleven *A. citrulli* and eleven saprophyte strains, collected from two naturally contaminated seed lots coming from China and Thailand, and sixteen plantlets showing typical symptoms from *A. citrulli* in a grow-out sweat box assay and three plants showing atypical symptoms in the grow-out assay were tested with the sweat box grow-out method. Only *A. citrulli* strains and plantlets showing typical symptoms gave a positive bioassay result, while saprophyte strains or plantlets showing atypical symptoms gave a negative result. The analytical specificity of the method was thus demonstrated.

Analytical sensitivity

The smallest amount of the target pathogen that can be detected, i.e. the limit of detection, is called analytical sensitivity. The sweat box grow-out assay should be able to detect one *A. citrulli* contaminated seed in one subsample (the least infected condition). To test this, one artificially contaminated seed was added to 14 subsamples of 799 healthy melon seeds. In two rounds of experiments testing seven of these subsamples at the same time, all 14 positive samples were observed out of the 14 expected for the least infected condition (one seed contaminated in 800 seeds).

In all tests, around 10% of the plantlets displayed typical symptoms on cotyledons, which means that neighbouring plants also became contaminated from secondary infection. The included healthy control did not display any symptoms. The sensitivity require-



Method process flow ISHI-Veg Sweat Box Grow-out method

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ments for the sweat box assay were therefore met.

Selectivity

With selectivity, the effect of different seed matrices on the ability of the method to detect the target pathogen is tested. The study of the matrix effect is of less relevance for a biological assay compared to, for example, molecular assays, since the seed background is less likely to interfere with the test result. The inter-laboratory comparative test (CT) was performed using two naturally contaminated melon seed lots, one 'yellow canari' type produced in 2012 in China, the other one a 'Piel de sapo' type produced in 2014 in Thailand. Seven laboratories tested 13 repeats of these two seed lots, named a highly infected and a moderately infected seed lot. The 13 repetitions of 800 seeds gave around 10,000 seeds tested, the recommended sample size to detect *A. citrulli* on melon seeds. All laboratories found the two contaminated lots (high and medium infection) to be positive for *A. citrulli*, meaning that the selectivity requirements for the method had been met. Due to the high genetic diversity between the two seed lots tested, no additional seed varieties were added.

Repeatability and reproducibility

As already mentioned under analytical specificity, it is essential to verify that the sweat box grow-out method gives reliable and consistent results when detecting *A. citrulli*. Samples for homogeneity, stability and CT were tested in one lab (the organising lab) and used to evaluate the repeatability of the method. In the CT, 13 repeats of three seed lots of 800 seeds each (highly infected, medium infected and healthy seed lots) were tested. These seed lots were characterized by the organizing laboratory as per the recommendation made by ISTA in its guidelines for organizing and analysing proficiency and comparative tests. In the homogeneity test, eight extra samples of 800 seeds representing each contamination level were tested after packaging and just before sending the samples to participating labs in the CT. In the stability test, three extra samples of 800 seeds representing each contamination level were tested after receiving the confirmation of all other participating labs in the CT that they started the test.

In the CT and stability tests, all healthy seed samples



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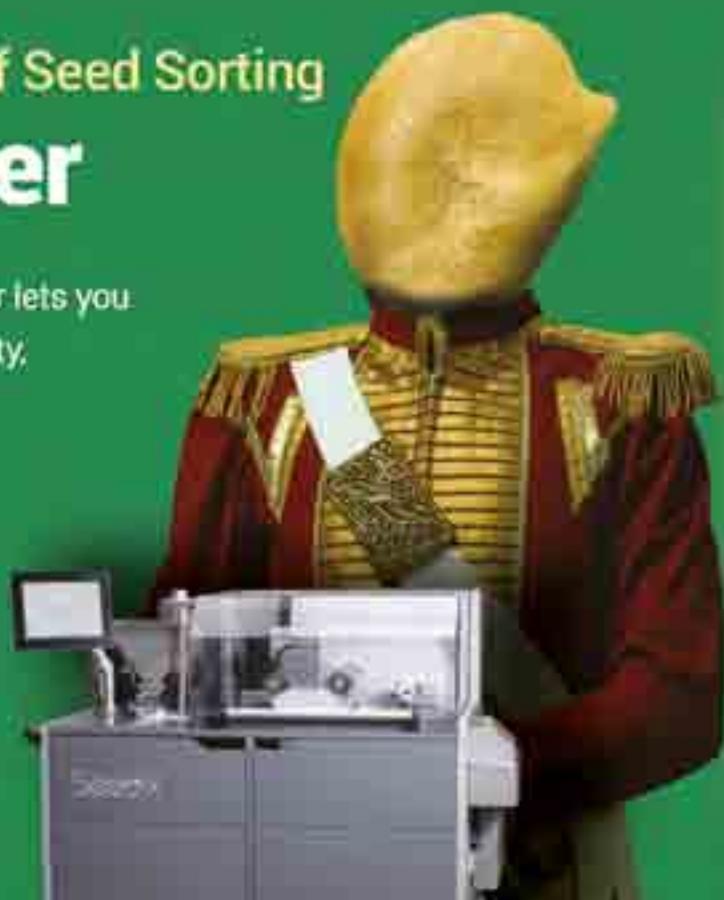
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Traditionally, the preferred method to detect *A. citrulli* was a seedling grow-out test

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gave a negative result, and all high infected seed samples gave a positive result which corresponds with the results from the homogeneity test. This results in a repeatability (accordance) for the healthy and high contaminated seed lots of 100%, using the method developed by Langton et al.

For the medium contaminated seed lot, the homogeneity test results showed that not all samples contained infected seeds. Here, the results from the CT and the stability test should fall within the expected number of contaminated samples, as calculated with the 'probability of k positive samples out of n' provided on the SHC ISTA webpage, based on the percentage of infection obtained from the homogeneity test, calculated with the Seedcalc8 software provided in the STATCOM ISTA webpage. The observed one positive sample in the stability test for the medium contaminated seed lot falls within the expected range of zero to three positives of the three samples tested. In contrast, the eight positive results obtained in the CT are above the expected range of two to seven positive samples. However, this difference was found not to be statistically significant when considering the overall CT data, as described below.

Inter-laboratory CT

Eight laboratories participated in the inter-laboratory CT, but only seven performed the bioassay due to

	Expected + result	Expected - result	Diagnostic sensitivity (TP / [TP + FP]) x 100%	Diagnostic specificity (TN / [FP + TN]) x 100%
Obtained + result	88 (TP)	0 (FP)	97.78%	100.00%
Obtained - result	2 (FN)	91 (TN)		
Total	90	91		

Analysis of qualitative inter-laboratory CT results for the healthy and highly contaminated seed lots. TP = True Positive, FN = False Negative, TN = True Negative and FP = False Positive.

a quarantine issue for one laboratory. The results from this laboratory were, therefore, excluded from the analysis. Each participating laboratory received 43 coded samples of 800 seeds each. Thirty-nine of them represented 13 repeats of three seed lots (highly infected, medium infected and healthy seed lots). The remaining non-coded seed samples (four) represented two repeats of 800 seeds each of the healthy and artificially contaminated seed lot and served as negative and positive process controls.

The result depended on the biological assay only. If one extract coming from one sweat box gave a positive bioassay, the sample was considered as positive. Participants reported a qualitative (positive/negative) result for each sample. Inconclusive (no clear typical symptoms in the bioassay) or undetermined (too much growth of saprophytes causing damping off) results were excluded from the statistical analysis. A reproducibility (concordance) for the healthy and high contaminated seed lots of 100%, and 95.6% respectively was calculated, using the method developed by Langton et al.

For the medium infected samples, concordance could not be calculated due to the homogeneity test results. Here, the expected number of positives samples according to infection rate is calculated per laboratory, with the 'probability of k positive samples out of n' and Seedcalc8 software.

Expected range

Three labs reported positive results for the medium contaminated seed samples which were within the expected range, while four labs reported more positive samples, as expected. The calculation of the expected number of positive samples for the medium infection level is based on the estimated level of infection which is calculated based on the results of the homogeneity test. The observed number of positive samples lies outside the expected range for four out of the seven labs, suggesting that the results of the CT do not match the expected range and hence, not the estimated level of infection. To test this hypothesis, the results of the CT were used to estimate the probability of a positive sample (of 800 seeds) and this estimate was then used to calculate the probability of finding three positive samples out of eight, the result of the homogeneity test. This probability was found

Analytical sensitivity test results

	Symptomatic plantlets	Rotten plantlets	Symptomless plantlets	% of contamination	Positive/tested samples
1 infected seed in 800 1 st experiment					
rep1	100	0	≈700	≈12.50%	
rep2	99	0	≈700	≈12.38%	
rep3	43	0	≈750	≈5.38%	
rep4	111	0	≈650	≈13.88%	
rep5	76	0	≈700	≈9.50%	
rep6	117	0	≈650	≈14.63%	
rep7	72	0	≈700	≈9.00%	
total	618	0	≈5000	≈11.04%	
rep1	80	0	≈700	≈10.00%	7/7
rep2	69	0	≈700	≈8.63%	
rep3	94	0	≈700	≈11.75%	
rep4	49	0	≈750	≈6.13%	
rep5	108	21	≈650	≈16.13%	
rep6	85	0	≈700	≈10.63%	
rep7	54	0	≈750	≈6.75%	
total	539	21	≈5000	≈10.00%	
Healthy control	0	0	≈1600	0.00%	

Qualitative results of the inter-laboratory comparative test

to be higher than 5%, a frequently used significance level, for which it is not significantly different from the expected results based on the CT test results. The repeatability (accordance) and reproducibility (concordance) of the method for the healthy and high contaminated seed lots are above 95%. The CT results observed for the medium contaminated seed samples fell outside the expected range of detection, but were found to be not statistically different from those obtained for the homogeneity test. Healthy and contaminated samples were, therefore, reliable, and consistently identified by the method. Therefore, the repeatability and reproducibility of the method was fit for purpose.

Diagnostic performance

To assess diagnostic performance, the results of the method should be compared to the true disease status of the seed lot. Diagnostic sensitivity and diagnostic specificity are measures of the test's ability to discriminate between the presence and absence of the target pathogen. Diagnostic sensitivity is the test's capacity to give a positive result when the target pathogen is present, while diagnostic specificity is a measure of the certainty that a negative result is a true negative.

For the medium infected samples, the expected number of positive samples according to infection rate was calculated (28-36 expected positive samples out of the 84 samples tested). The actual detected samples, 54 positive samples, fall outside this expected range. This correlates with the repeatability and reproducibility results, which showed that four labs detected more positive samples from the medium contaminated seed lot, as expected. As mentioned above in the reproducibility section, this

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was found not to be statistically significant. The diagnostic sensitivity (percentage of samples correctly identified as positives) and the diagnostic specificity (percentage of samples correctly identified as being negative) of the method for the healthy and highly contaminated seed lots are above 95%. The method, therefore, confirmed healthy samples and detected highly contaminated ones. The results observed for the medium contaminated seed samples fell outside the expected range of detection. However, although a higher number of positives than expected were observed, the difference was found not to be statistically significant. The diagnostic performance was therefore fit for purpose.

Suitable method

The performance criteria assessed during method validation confirm that the ISHI-Veg sweat box grow-out method for the detection of *A. citrulli* from melon seeds is suitable to detect contaminated seed lots with viable and infectious *A. citrulli* bacteria in melon. All participating laboratories in the inter-laboratory CT found the healthy lot to be healthy and the two contaminated lots (high and medium infection) to be positive for *A. citrulli*. The 13 repetitions of 800 seeds gave around 10,000 seeds tested, the recommended sample size to detect *A. citrulli* on melon seeds. Training on the method before routine use is recommended.

The validation was performed on melon seeds only and it is the user's responsibility to validate the results obtained in this study prior to the test being used for other host seed species. The ISHI-Veg International Technical Group Cucurbits will work on the validation of the sweat box grow-out method for watermelon in 2021/2022.

The ISHI-Veg protocol on 'The detection of *A. citrulli* associated with melon seeds by grow-out in sweat boxes', together with the validation report, was first published online in May 2021 and can be found on the ISF website (<https://www.worldseed.org>). 

The validated protocol on this assay was first published online in May 2021 by the International Seed Federation (ISF). Training on the method before routine use is recommended. (<https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-protocols/>)



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