Advisory Committee on Releases to the Environment

Advice on an application for deliberate release of a GMO for research and development purposes

Applicant: Cambridge University Crop Science Centre

Application: To undertake a field trial to investigate the impacts of arbuscular mycorrhizal fungi (AMF) inoculation on biomass and yield of both gene-edited and genetically modified symbiosis pathway genes in spring barley in low and rich phosphate soils.

Reference: 21/R54/01

Date: March 2022

Advice of the Advisory Committee on Releases to the Environment to the Secretary of State under section 124 of the Environmental Protection Act 1990

The Advisory Committee on Releases to the Environment (ACRE) is satisfied that all appropriate measures have been taken to avoid adverse effects to human health and the environment from the proposed release. ACRE sees no reason for the release not to proceed according to the following advice.

To minimise the likelihood that GM barley from this trial will enter the human food or animal feed chains, the applicant should:

1. Ensure that the 20 m surrounding the trial site is planted with a non-cereal crop and that cereal volunteers are controlled (prior to flowering) in this area during the trial.

2. Plant a barley pollen barrier, of 3 m width, to flower at the same time as the GM barley as an additional precautionary measure.

3. Control cereals and grass species using a glyphosate herbicide and hand weeding, if necessary, within the trial site and the surrounding 20 m, before flowering and for the duration of the trial.

4. Ensure that any GM or non-GM barley plant material remaining in the area of release at the end of the trial is disposed of appropriately.

5. Ensure that following harvest, the area of release is lightly tilled twice (once after harvest and again in the following spring) to a depth of 5 cm to stimulate germination of any barley plant volunteers. The release areas should be left fallow and monitored for barely plant volunteers for 2 years following harvest.

6. Record the number of barley plant volunteers that germinate before destroying them with an application of glyphosate herbicide or hand pulling them prior to flowering.

7. Ensure that suitable measures (such as those described in the Cambridge University Crop Science Centre's application) are put in place to keep large birds out of the trial area and that the efficacy of these measures are kept under review.

8. Ensure that machinery used on the site is cleaned thoroughly onsite, including between using it with GM and non-GM material, and that clothing and equipment such as vehicles used by personnel on the site are also cleaned thoroughly before leaving the site.

Comment

ACRE considered the risks to human health and the environment posed by the proposed release of barley that has been both gene edited and genetically modified with respect to symbiosis pathways to investigate the impacts of arbuscular mycorrhizal fungi (AMF) inoculation on biomass and yield in the field under both low and rich phosphate soils.

The release will use 11 independent gene-edited lines, each of which contains insertions/deletions (indels) in one of the six genes involved in perception of AMF. These lines revealed significant reduction in AMF colonisation under laboratory conditions.

The release will also involve two further lines that have been genetically modified via the introduction of T-DNA containing one of the afore-mentioned six genes (namely, NSP2) either from *Hordeum vulgare* or its homologue from *Medicago truncatula* such that it is overexpressed. These two lines promoted AMF colonisation in controlled environmental conditions.

Key characteristics of this field trial with respect to its environmental risk assessment are:

i) It will be on a small scale. This application is to release approximately 300 seeds per m^2 over an area of 2100 m^2 comprised of any eight of the thirteen GE/GM barley lines and one control line making for a maximum of 183,600 GM plants being grown each year. The proposed release will be conducted within the agricultural areas of the experimental farm sites managed by NIAB's Cambridge Regional trials centre, Cambridge, UK. The applicant has proposed that the release will take place at different locations within the regional trials centre over five years. The trial will be planted first in the spring of 2022 and then sequentially until the final harvest in autumn of 2026.

ii) The GM barley and non-GM barley grown in this trial will not be put into the human food chain or fed to livestock.

The applicant intends to trial a total of 13 transgenic lines; two of these contain gene constructs. These GM lines may also contain the antibiotic resistance genes encoding neomycin phosphotransferase I (*npt1*) and hygromycin phosphotransferase (*hyg*). NPT1 confers resistance to aminoglycoside antibiotics such as kanamycin and neomycin. These genes are used in the development of GM plants to facilitate the selection of bacteria and plants (respectively) that have been transformed successfully.

Molecular Characterisation

Cambridge University Crop Science Centre (CU-CSC) have individually edited six of the barley cv. Golden Promise genes involved in the perception of AMF, namely SymRK, CCamK, Cyclops, RAM1, NSP1, and NSP2 using the CRISPR-Cas9-mediated gene editing system.

The application outlines in detail the sets of guide RNA (gRNA) used to target the above genes, and which resulted in eleven gene-edited lines. Similarly, the methodology followed to make these lines, their subsequent molecular and contained use phenotypic characterisation, and the generation of two further lines transformed to contain inserted genes is also described.

ACRE noted that both the gene editing and genetic modification was made using *A. tumefaciens* mediated transformation to incorporate either the CRISPR-CAS gene editing genes or the NSP gene from either *H.vulgare* or *M.truncatum*, along with constitutive expression promoters, into a nuclear location.

Although the insertion sites were not characterised in molecular detail, but there has been phenotypic analysis, and this will be a small-scale trial where plants won't enter into the human food chain. Therefore, ACRE concluded that in the case of this particular trial, additional data on molecular characterisation would not be helpful in addressing risk-based questions, and that the molecular characterisation was perfectly adequate. Although the application did not contain the actual data that they used, ACRE were content with the proposers' statements that they screened for the loss of T-DNA and other elements in the gene edited lines within the segregated plants as there were no reasons to suspect that there may be a problem.

ACRE recognised that it was impossible to assert that there have been no off-target effects caused by the gene editing process, but there was no evidence from the phenotypic data generated so far to suggest that these had any impact, if they have occurred at all. However, it was conceivable that the field trials might reveal phenotypic differences not seen in the work done to date that could be caused by off-target events. In that eventuality even if these were to have an impact on the environment, the proposed trial had procedures in place for terminating the trial, and for ensuring that no plants persist long term.

ACRE further concluded that the same argument applied regarding the two GM lines. The exact location of where the cassettes were, or their overall integrity was not clear, but again there were no phenotypic effects, and this was a small-scale trial with no likelihood of material entering the food/feed chains. ACRE therefore did not see that any further characterization was needed at this stage, to inform the environmental risk assessment.

ACRE considered further the implications of the inserted gene cassette including that of the presence of two selectable marker genes, and on the basis that the nptI marker gene is outside the T DNA right and left borders, the CU-CSC's statement that it was not present in transformed plants was correct. The structure of either gene and their associated promoters in the T-DNA cassette was standard for experiments of this type and therefore ACRE did not conclude that there were any further molecular aspects to be concerned about.

Some of the public representations criticised CU-CSC's molecular characterisation of the GM lines for not including information on unintended effects on the genome, including the role this may play in altered phenotype of the resultant plants. This has been discussed above and suffice to say that these data are not required in applications for small trial releases of GM plants unless they are needed to inform the risk assessment. ACRE were reminded of their previous discussions on what intrinsic characteristics of cereals these (or other) alterations would need to change in order for them to confer an environmental risk e.g., to make barely a problem weed[1].

It is inevitable that there will be differences between plant lines, including those that have been gene edited. This is the case for conventional plant breeding as much as it is for either GM or GE. Attempting to interpret these differences is challenging and not constructive unless there is an indication of what hazard to look for. Under controlled conditions, both the GE and GM plants are indistinguishable from untransformed plants. An objective of the trial is to determine whether this is the case under field conditions. Monitoring of GM plants is a standard requirement in any consent that is issued for a GM field trial.

Both ACRE and Public representations asked for information with respect to a remark made by the applicant that "the GE and GM barley genotypes exhibit a difference in the expression pattern of a number of genes involved in the biosynthesis of plant metabolites. None of these **genes** are known to be toxic or harmful to human health, nor are they known to exert any toxic or allergenic

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[1] Chepil W.S. (1946) Germination of Weed Seeds I. Longevity, Periodicity of Germination, and Vitality of Seeds in Cultivated Soil. Scientific Agriculture **26**: 307-346.
Anderson, R. L. and G. Soper. 2003. Review of volunteer wheat (Triticum aestivum) seedling emergence and seed longevity in soil. Weed Technol **17**:620–626.

effects.” (The word in bold should be “metabolites”). CU-CSC responded with an addendum to this section of the application, including diagrams of the biosynthetic pathway that results in those metabolites, and of their altered levels as measured in both GE and GM barley lines. The end products of these enzymatic pathways are strigolactones; which are naturally produced by plants to promote growth of AMF and hence help establish symbioses with plants. ACRE were content with this clarification, and with the CU-CSC conclusion that these metabolites were not known to be toxic or harmful to human health.

The Environmental Risk Assessment

ACRE members discussed whether the phenotype of the GM lines, namely enhanced AMF colonisation in high phosphate soils, was a trait of potential concern in terms of its ecological impact. Members thought that the benefit of this approach hinged on the carbon budget in the plant; that is at high Phosphate levels was it cheaper for barley to grow roots or to use fungi to harvest phosphorous? Recent work in maize suggested that there was less carbon cost to get fungi to do the work even at high Phosphate.[2] However, this may not be true of barley as the authors recognised that there was limited data on other crops.

ACRE concluded that the aim of CU-CSC in this trial was to see if high AMF colonisation led to more or bigger seeds as a result of more efficient carbon allocation. if it did then that was one measure of plant fitness, and it would result in an increase under high Phosphate. However, ACRE were content that this would not significantly impact on the ecology of barley volunteers or affect barley's invasiveness in natural or semi-natural habitats where we would not expect high levels of soil phosphorous. Although, ACRE were also cognisant of the containment measures put in place by CU-CSC/NIAB that would adequately address any such increased risk.

Barley is naturally self-pollinating but under experimental conditions can be crossed with various wild grasses. The application discusses sexual compatibility with wild relatives such a, *Hordeum spontaneum* (wild barley) and *H. bulbosum* (bulbous barley). Both of which, CU-CSC points out, are restricted to south-eastern Europe, North Africa and Asia and that no reports of hybridisation with cultivated barley have been identified in the UK. However, CU-CSC plans to monitor to ensure that no cereals or grass species will be allowed to grow within 20 m of the trial area itself.

There is within the application an assessment of the likelihood of horizontal transfer of the gene cassette present in the GM lines and specifically of the antibiotic resistance genes, along with consideration given to recombination with soil bacteria. Furthermore, some public representations reflected concern

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[2] Ven, A., Verlinden, M., Verbruggen, E. and Vicca, S. (2019) Experimental evidence that phosphorus fertilization and arbuscular mycorrhizal symbiosis can reduce the carbon cost of phosphorus uptake. Functional Ecology **33** (11): 2215-2225.

that growing plants containing antibiotic resistant marker genes would compromise the use of associated antibiotics in human and veterinary medicine. ACRE has discussed the use of resistance marker genes in GM plants on a number of occasions and taken into consideration the statement from the European Medicines Agency (EMA) on the importance of preserving the therapeutic relevance of the antibiotics.

ACRE emphasised that both the *nptI* and *hyg* genes are present at high frequency in agricultural soils[3]. (Although the *nptI* gene is, for reasons discussed under molecular characterisation, unlikely to be present in the transformed plants). Antibiotic resistant bacteria occur naturally in the environment, but many are a result of contamination with human and animal excreta in sewage, slurry and manure. Antibiotic resistance in humans and other animals has resulted from the strong selective pressure associated with the substantial use of industrially made antibiotics in human and veterinary medicine and as food supplements for farm animals.

To add to the above, ACRE gave the following advice on plant to bacterial gene transfer in a previous field trial application:

Even though the scientific consensus is that selection pressure on bacteria containing antibiotic resistance genes is the driver of antibiotic resistance gene frequency in the environment, ACRE discussed the potential for bacteria in the environment to be transformed with antibiotic resistance genes from the genetically modified barley plants. Studies of horizontal gene transfer from plants to bacteria suggest that this phenomenon is extremely rare (Please refer to a review by Keese, 2008[4]).

ACRE noted that even if a recombination event were to occur between DNA from a plant and a bacterial genome, in order for the gene to be expressed, it would need to be combined as a fully functional transcription unit in the bacterium, which is unlikely. If it were to occur, it would most likely result from a homologous recombination event at a site in the bacterial genome where a version of antibiotic resistance gene already exists.

Managing the trial site

The area for the proposed trial will comprise 2100 m^2 total area per year (comprised of 108 plots each of 15. X 4.25 metres) that will be sown at a density of 300 seeds m^2. There will be 1.0 m separation between plots and a barley pollen barrier of 3 m width entirely surrounding the trial plots, with a further 20 m surrounding that, in which no cereals or grass species will be left to grow.

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[3] Walsh F, Duffy B (2013) The Culturable Soil Antibiotic Resistome: A Community of Multi-Drug Resistant Bacteria. PLoS ONE **8**: e65567.
[4] Keese P. (2008). Risks from GMOs due to horizontal gene transfer. Env Biosafety Research. **7**(3): 123 – 149

ACRE has considered the potential risks of this trial to human health and the environment in the context of it being a small-scale trial from which no material will enter the food or feed chains, the committee considered, in detail, management plans to minimise the persistence of GM material at the trial site and the dispersal of GM material from the site. ACRE recognised that, although the proposed trial was larger than a previous trial (albeit of wheat) in terms of area, a larger, 3 m pollen barrier + 20 m isolation distance was adequate to minimise the probability of out-crossing to an acceptable degree.

The barley pollen barrier will be in place and so designed to flower at approximately the same time as the GMO crop, but ACRE acknowledge that this is not an exact science. However, the trial is further contained by a surrounding 20 m isolation distance and the probability of crossing with wild species is very low/negligible. The GE/GM plants are susceptible to a wide range of herbicides and therefore, if necessary, it will be straightforward to kill off these plants. ACRE considered that the post-harvest processing protocol was robust, and that the described trial management procedures reflected the level of experience that NIAB have in handling GM trials.

ACRE requested that the applicant consider how best to prevent seeds being taken by birds and small mammals, as the original application merely referred to the use of bird scarers. In response CU-CSC altered their site management plans to include the use of full-height framework and netting in addition to bird-scaring devices during the growing season

Gene flow

Barley is a self-pollinating crop with very low rates of cross-pollination with other barley plants. This is because fertilization often occurs before the florets open, which makes out-crossing unlikely; in addition, barley pollen is relatively heavy and tends to travel shorter distances than pollen from other grass species that are wind-pollinated. Studies have detected cross-pollination rates of 1 to 7% between barley plants in close proximity, but this rapidly decreases with the distance between plants[5]. There are several relevant studies involving GM cereal field trials, most recently those of Foetzki *et al*. (2012)[6] and Miroshnichenko *et al.* (2016)[7] .

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[5] Ritala A., Nuutila A., Aikasalo R., Kauppinen V. and Tammisola J. (2002). Measuring gene flow in the cultivation of transgenic barley. Crop Science **42**(1), 278-285.

[8] Foetzki A., Diaz Quijano C., Moullet O., Fammartino A., Kneubuehler Y. and Mascher F. (2012). Surveying of pollen-mediated crop-to-crop gene flow from a wheat field trial as a biosafety measure. GM Crops and Food: Biotechnology in Agriculture and the Food Chain **3**(2), 115–122.

[7] Miroshnichenko D., Pushin A and Dolgov S (2016). Assessment of the pollen-mediated transgene flow from the plants of herbicide resistant wheat to conventional wheat (*Triticum aes. Gene flow from transgenic wheat and barley under field conditionstivum* L.). Euphytica **209**:71–84.

ACRE noted that the separation distance required to prevent hybridisation between different barley varieties when certified seed is produced for marketing purposes is 2 metres. The application proposes to sow a 3-metre-wide barley pollen barrier (comprising the same variety as the GM barley) around the trial. ACRE recommended a 2-metre-wide pollen barrier in its advice on previous GM wheat trials as this is an additional precautionary measure to the 20-metre separation distance. But, recognising the larger size of this trial, ACRE sees no reason to extend this to ensure an acceptable probability of no unacceptable gene flow. In order to maintain the separation distance, ACRE advises that the 20 m surrounding the trial site is planted with a non-cereal crop and that cereal volunteers are controlled (prior to flowering) in this area during the trial and for two years afterwards.

ACRE members considered that in terms of the pollen barrier, the key was timing to make sure both the experimental crops and the pollen barrier crop were at the same stage of development. That can be difficult if one is looking at experimental seed that does not have all the characteristics and stability of a commercial variety. The committee concluded that, in their view, if synchronisation proves difficult, then the 20 m separation distance would be an acceptable risk mitigation.

The applicant plans to move the specific site around within and between the separate sites that form part of the NIAB Cambridge Regional trials centre to allow post trial monitoring and to avoid the effects of take-all disease on the plants. Therefore, the location of the specific growing site will require careful consideration to ensure that the 20 m isolation distance remains within the bounds of the NIAB regional trials land as a whole.

Public representations were received that pointed to larger isolation distances being needed in order to prevent cross-hybridisation between cereal crops. However, there is considerable data on the effect of a pollen barrier in drastically reducing the size of such isolation distances. There are a number of published studies on gene flow from transgenic crops, both with and without pollen barriers, and in European climates. One such study used field sizes of 100 x 100 m = 10 000 m^2 = 1 ha (by comparison the CU-CSC is only c. 2000 m^2 so 1/5 ha) and found gene flow in transgenic barley to occur at very low frequencies and over short distances. So much so that isolation distances of 12 m resulted in contamination levels of less than 0.02% in adjacent cereal crops (Gatford et al. 2006)[8].

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[8] Gatford K., Basri, Z., Edlington, J., Lloyd, J., Qureshi, J., Brettell, R. and Fincher, G. (2006).
Gene flow from transgenic wheat and barley under field conditions. Euphytica **151**:383-391.

Barley plant volunteers

The trial will receive standard farm practice as regard to herbicides, fungicides, nitrogen, sulphur and other fertilisers.

The site will be monitored regularly (at least weekly) both during and for two years after the trial. For the Post- trial monitoring period, the trial area will remain in stubble to enable monitoring of volunteers. The applicant mentions shallow cultivation being carried out to encourage volunteers and specifies that this will be carried out in both spring and autumn. This is in agreement with previous ACRE advice on that trials sites should be lightly tilled twice (once after harvest and again in the following spring) to a depth of 5cm to stimulate germination of any wheat plant volunteers. The persistence of such volunteers from cereal crops in cultivated soil has been studied for a long time and is well-characterised. [9],[10]

There are several relevant publications, of which the most detailed are two specifically designed to consider longevity of cereals in the seed bank in the context of GM (Kristi *et al*. 2007[11] and Ryan *et al.* 2009[12]). These studies conclude that survival of buried seed beyond the next spring is extremely rare and longer-term persistence in a field is most likely to occur from seed produced from volunteers that escape detection in the following season and then set seed. This conclusion is supported by the more recent study by Kalinina *et al*. in 2015[13].

ACRE recognised that conventional barley crops can give rise to volunteers within agricultural fields but are rarely invasive outside of these environments. Furthermore, barley volunteers are only a problem to control in another cereal crop such as wheat since herbicides are not sufficiently discerning to kill only the barley volunteers. Therefore, in non-cereal-based crops or in fields that

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[9] Chepil W.S. (1946) Germination of Weed Seeds I. Longevity, Periodicity of Germination, and Vitality of Seeds in Cultivated Soil. Scientific Agriculture **26**: 307-346.

[10] Anderson, R. L. and G. Soper. 2003. Review of volunteer wheat (Triticum aestivum) seedling emergence and seed longevity in soil. Weed Technol **17**:620–626.

[11] Kristi A. De Corby, Rene C. Van Acker, Anita L. Brûlé-Babel, and Lyle F. Friesen (2007). Emergence Timing and Recruitment of Volunteer Spring Wheat**.** Weed Science **55**(1): 60-69.

[12] Ryan L. Nielson, Marc A. McPherson, John T. O'Donovan, K Neil Harker, Rong-Cai Yang, and Linda M. Hall (2009). Seed-Mediated Gene Flow in Wheat: Seed Bank Longevity in Western Canada. Weed Science **57**(1): 124-132.

[13] Olena Kalinina, Simon L. Zeller, Bernhard Schmid (2015). Persistence of seeds, seedlings and plants, performance of transgenic wheat in weed communities in the field and effects on fallow weed diversity. Perspectives in Plant Ecology, Evolution and Systematics **17**: 421–433.

are going to be left fallow, control can be achieved using a specific grass-based herbicide or a broad-spectrum herbicide. ACRE concluded that as the fields in the proposed GM trial are going to be left fallow following the end of the experiment for a monitoring period, they did not consider barley volunteer control to be an issue and the existing containment measures are appropriate

Seed movement

ACRE were content with the applicant's outline of how the release will be monitored regularly during all stages of development and harvested at maturity. Some seeds from the GM and control plots will be conditioned, threshed and stored in appropriate GM seed stores. A sample of plants may be hand-harvested, conditioned and threshed to supply seeds for research purposes. All such small samples removed from the trial site will be stored in containment prior to use and will eventually be autoclaved before disposal. The remainder of the site will be harvested by the plot combine.

Grain that is not required for analysis or to provide seed for future trials and all other material, including that from the pollen barrier rows, will be disposed of by incineration, autoclaving, or deep burial at a local authority-approved landfill site using an approved contractor, while any material remaining after analysis will be autoclaved before disposal. Transportation of waste materials will be in secure containers. All straw will be chopped and left on site. The combine will be cleaned prior to leaving the site so that all traces of plant material from the trial will remain in the trial area. All transport of material will be logged.

Other items arising from public representations

Some 114 public representations were received, where these covered areas within the remit of ACRE they were addressed within its assessment, as summarised above. Many of the representations concerned areas beyond the remit of ACRE, and/or these topics are not relevant to the environmental risk assessment when considering a small-scale field trial. Some of these comments would be of relevance if the application had been for commercial-scale cultivation and/or food and feed use. Others were more political in nature and are outside of ACRE's remit; for example, they questioned whether GM techniques should be used in the development of plant varieties in general and more specifically whether it is necessary to develop barley with enhanced AMF interaction using this technology.