STATEMENT

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Statement on the translocation potential by *Pseudomonas chlororaphis* MA342 in plants after seed treatment of cereals and peas and assessment of the risk to humans

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Abstract

The European Commission requested EFSA to provide scientific advice on the translocation potential by *Pseudomonas chlororaphis* MA342 in plants after seed treatment of cereals and peas and, if applicable, for a revision of the assessment of the risk to humans by its metabolite 2,3-deepoxy-2,3-didehydro-rhizoxin (DDR) and this based on the evidence available in the dossier for renewal of the approval. The information from other *P. chlororaphis* strains than MA342 was taken into account with care, because the studies available in the dossier did not confirm the identity of the strain MA342 as belonging to the species *P. chlororaphis*. It has been concluded that there is a potential for translocation of *P. chlororaphis* MA342 to edible plant parts following seed treatment till an estimated concentration up to about 10^5 cfu/g and some exposure can be assumed by consumption of fresh commodities. Also, production of the metabolite DDR in the plant cannot be excluded. Regarding levels of DDR in the raw agricultural commodities, exposure estimates based on the limit of quantification (LOQ) for DDR in cereals cannot be further refined while there is no information on the levels of DDR in peas in the dossier. As regards genotoxicity, DDR induced chromosomal damage; however, it was not possible to conclude whether it is through an aneugenic or clastogenic mechanism. Hence, it is not possible to draw a reliable conclusion that DDR is producing an aneugenic effect nor to determine a threshold dose for aneugenicity. Thus, it is not possible to revise the human risk assessment as regards exposure to DDR. The concerns identified in the EFSA conclusion of 2017 remain.

Keywords: *Pseudomonas chlororaphis* MA342, metabolite, DDR, genotoxicity, aneugenicity, seed treatment, cereals, peas, translocation, human risk assessment

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Summary

Pseudomonas chlororaphis strain MA342 is an active substance approved in EU since 2004 as a fungicide for seed treatment. In the EFSA conclusion on the peer review for the renewal of the approval of *P. chlororaphis* strain MA342 (2017) critical areas of concern as regards a risk for consumers from exposure to the genotoxic metabolite 2,3-deepoxy-2,3-didehydro-rhizoxin (DDR) were identified. The European Commission noted that the decision on the first approval was based on the opinion of the Scientific Committee of Plants, which had concluded that residues had been adequately addressed and no concern had been identified. In the renewal evaluation, there were divergent opinions of the rapporteur Member State, co-Rapporteur Member State, Member States' experts and EFSA related to the interpretation of some studies. Given that the issue is critical for decision-making, the European Commission asked EFSA to review all relevant data on *P. chlororaphis* strain MA342 available in the renewal dossier, and to provide an assessment of the capacity of *P. chlororaphis* MA342 for translocation to plant parts and proliferation after seed treatment of cereals and peas and to consider if it is possible to complete and/or refine the assessment of the exposure of consumers, workers and residents to metabolites produced by this microorganism (European Commission, 2020). Further consideration on the aneugenicity of the metabolite DDR (see Appendix A) and confirmation whether or not it is possible to set a no observed effect level (NOEL) for the genotoxic potential was also requested.

The PPR Panel interpreted that the terms of reference require the Panel to provide advice on:

- Translocation of the microorganism to edible parts of the plants and production of DDR there.
- Uptake of the metabolite DDR from soil and transport to the edible parts of the plants.
- Aneugenicity of metabolite DDR.
- Possible refinement of risk assessment for consumers, workers and residents to metabolites of *P. chlororaphis* MA342.

For this statement, the PPR Panel has considered all relevant evidence and information available in the dossier for renewal of the approval.

The main conclusions are presented below:

Many of the studies had limited reporting quality, were performed with other species or strains, were found to be not reliable or were incomplete and important information was missing in the dossier.

There are no data available in the dossier confirming the identity of the strain designed as *P. chlororaphis* MA342 as belonging to the species *P. chlororaphis*. This is a source of uncertainty in relation to the relevance of data of studies referring to only the species, not mentioning the specific strain MA342. Therefore, these studies were taken into account with care.

Based on the studies in the dossier, supported by literature data, it can be assumed that *P. chlororaphis* MA342 used as a biocontrol agent by seed treatment, could be present internally in the edible plant tissues. Based on these data, the internalised bacteria can survive, translocate in the plant and grow till an estimated concentration up to about 10^5 cfu/g. The potential production of DDR by the microorganism within the plant cannot be excluded. With the information available in the dossier, it is not possible to make a quantitative estimation of possible DDR production from the amount of microorganism in the plant.

It is not possible by simply washing edible plant parts to remove attached or internalised bacterial cells. Therefore, some exposure to *P. chlororaphis* MA342 has to be assumed by consumption of fresh commodities. However, the processing of these raw commodities with methods involving sufficient heating (e.g. by cooking or baking processes for sufficient time/temperature combination) will result in the death of the bacteria.

There is limited or no information in the dossier regarding the production of DDR or other relevant metabolites by *P. chlororaphis* MA342 during different growth stages. No information on DDR background levels or its production in soil, uptake by plants and transport to other plant parts is available. Therefore, it is not possible to confirm or exclude the possibility of residues of DDR in plant commodities coming from its occurrence/production in soil or the plant. There were no field residue trials investigating the viable and non-viable residues of *P. chlororaphis* MA342 in cereals or peas at harvest. Studies in greenhouses analysing the content of DDR in different plant parts grown from treated cereal seeds showed that the levels were below the limit of quantification (LOQ $< 1–3$ μg/kg). The reliability of these studies was, however, considered to be low. Whereas the PPR Panel considers it highly likely that DDR will degrade to a certain extent before consumption, no relevant data (e.g.

metabolism and processing studies) are available in the dossier to quantitatively estimate the extent of this potential degradation. The overall conclusion is that DDR may be present in the edible crops only at low levels, but it is not possible to conclude at what specific amount. Therefore, regarding levels of DDR in the representative commodities, it was concluded that exposure estimates based on the LOQ for DDR in cereals (< 1–3 μg/kg) cannot be further refined with the information available in the dossier. For peas, there were no studies or information in the dossier regarding residue levels of DDR.

As regards the genotoxicity of DDR, the PPR Panel consulted the Standing working group on genotoxicity of the EFSA Scientific Committee for advice on aneugenicity of the metabolite DDR, since a Guidance on aneugenicity assessment is currently in a late stage of development. The two available genotoxicity studies were re-assessed and it was confirmed that DDR induces chromosomal damage; however, it is not possible to draw a reliable conclusion on an aneugenic effect or to determine a threshold dose for aneugenicity.

Since neither the exposure estimate nor the toxicity endpoint of the metabolite DDR can be refined with data available in the dossier, the PPR Panel concludes that it is not possible to revise the risk assessment for the consumers or complete the risk assessment for workers and residents exposed to DDR based on the information in the dossier. Thus, the concerns identified in the EFSA conclusion of 2017 remain.

To reduce uncertainties in the current assessment, recommendations were made on how to collect data on exposure to *P. chlororaphis* MA342 and its metabolites and on the further genotoxicity testing of metabolite DDR.

To finalise the risk assessment on *P. chlororaphis* MA342, a proper taxonomic characterisation should be performed and data on the antimicrobial production and antimicrobial resistance should be envisaged. As *P. chlororaphis* is not recommended for the Qualified Presumption of Safety (QPS) status, a risk assessment of the presence of *P. chlororaphis* strain MA342 in edible plant tissues would be necessary.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

Pseudomonas chlororaphis strain MA342 is an active substance included in 2004 in Annex I to Council Directive 91/414/EEC as a fungicide for seed treatment (field-grown monocotyledonous crops) in closed seed dressing machinery.

P. chlororaphis strain MA342 is a microorganism which "is able to colonize soil, roots and shoots, but mainly the rhizosphere of plants. During plant development, bacterial population densities decrease strongly, especially in distances > 5 cm from seeds" as indicated in the List of Endpoints of EFSA Conclusion (Appendix A, page 2) (EFSA, 2017).

An application for the renewal of the approval of *P. chlororaphis* strain MA342 was submitted by Lantmännen BioAgri AB. In addition to use in seed treatment of cereals, the applicant proposed 3 other representative uses: seed treatment of carrots, seed treatment of peas and foliar application in cereals.

For *P. chlororaphis* strain MA342 the rapporteur Member State was The Netherlands and the co-rapporteur Member State was Denmark. In December 2015, The Netherlands finalised a renewal assessment report which included a recommendation to renew the approval of *P. chlororaphis* strain MA 342 for the supported uses (The Netherlands, 2016).

The conclusion on the peer review of the pesticide risk assessment of the active substance *P. chlororaphis* strain MA 342 was adopted by EFSA on 5 December 2016 (EFSA, 2017).

EFSA identified a critical area of concern as regards a risk for consumers from exposure to the genotoxic metabolite 2,3-deepoxy-2,3-didehydro-rhizoxin (DDR, see appendix A).

Critical area of concern - consumer exposure to genotoxic metabolite DDR

EFSA considers that DDR should be classified as mutagen category 2, genotoxic (aneugenic *in vitro* and *in vivo*). The NOEL proposed by the applicant was rejected during peer review process and the threshold of toxicological concern (TTC) value for genotoxic compounds was applied for the risk assessment. In the studies submitted by the applicant levels of DDR above 1 μg/kg were not found on edible parts of plants following seed treatment. The consumer exposure to DDR was therefore estimated assuming the presence of DDR at the LOQ (Limit of Quantification of available analytical method of 1 μg/kg). The calculated exposure of consumers was above the TTC for genotoxic compounds.

According to the EFSA Conclusion (page 16), "a refinement of the consumer risk assessment is currently not possible in the absence of specific toxicological reference values for the genotoxic metabolite DDR. A first-tier assessment using the TTC approach failed to demonstrate consumer safety".

Degradation of DDR

Furthermore, information on the persistence of metabolite DDR was found not reliable by EFSA.

On the other hand, an opinion of the Scientific Committee of Plants adopted in December 2001 (European Commission, 2002) had considered the claims on the low persistence of metabolite DDR for the assessment.

In the context of the decision making process on the renewal of approval, the ability to confirm the rapid degradation of DDR would imply the possibility for refinement of the consumer exposure to DDR residues.

P. chlororaphis capacity to translocate from treated seeds to crops - critical for decision making

The critical area of concern in the Conclusion of EFSA is directly related to the potential levels of the metabolite DDR on plant parts and to its toxicological properties.

Furthermore, as stated on page 9 of the EFSA Conclusion "Seed treatment uses are assumed less critical taking into account the much longer period between sowing of treated seeds and harvest of the mature crops as well as growth dilution of potential residues of DDR." Nevertheless, the EFSA Conclusion indicates also that: "This assumption can only hold true if it can be conclusively demonstrated that *P. chlororaphis* strain MA 342 does not have the capacity to translocate to plant parts after seed emergence and to proliferate, which is currently not the case."

Consequently, the capacity of *P. chlororaphis* to translocate to other plant parts after seed emergence and to proliferate appears to be an important element for the assessment of the consumer risk, and for the decision on renewal of approval (including for a decision on possible restrictions of approval to the least critical uses).

Studies addressing *P. chlororaphis* mobility - divergent interpretations

The reports dealing with the mobility of *P. chlororaphis* submitted by applicant for the purpose of the renewal (Anonymous, 1993a,b, 1997a,b, 1998b) were already considered in the course of the process for the first approval of the active substance. The decision on approval and inclusion in Annex I of Council Directive 91/414/EEC of *P. chlororaphis* MA 342 was based on the opinion of the Scientific Committee of Plants[1] as referred to in the Review Report of 2004 (European Commission, 2004).[1]

The same reports were not accepted as reliable by EFSA in the renewal evaluation of 2017 and were considered only as supporting information by the RMS. According to the EFSA Conclusion, in those reports "it is claimed that *P. chlororaphis* strain MA 342 is able to translocate to plant parts, especially to the coleoptile. (…) However, fully reliable studies investigating the presence of *P. chlororaphis* strain MA 342 in other plant parts and different growth stages are not available. Therefore, it is still uncertain to which extent *P. chlororaphis* strain MA 342 can translocate from the roots to other plant parts.

A data gap has been identified to provide information in relation to the mobility of the microorganism in the environment".

Nevertheless as mentioned above, when describing the biological properties of the microorganism, in the List of Endpoints of the EFSA Conclusion, 2017 (Appendix A, page 2) it is stated that "*P. chlororaphis* MA 342 is able to colonize soil, roots and shoots, but mainly the rhizosphere of plants. During plant development, bacterial population densities decrease strongly, especially in distances > 5 cm from seeds".

Given the ambiguity, and that the issue is a critical element for decision making, the Commission asked EFSA for clarification on the reasons to reject the studies submitted to address the mobility of *P. chlororaphis* and/or to interpret them differently than in the original evaluation.

In a reply to the Commission enquiry, EFSA prepared the annotated conclusion in which the studies relied upon and the deficiencies in some of these studies were highlighted. Additionally, EFSA provided the original reports to help the Commission understand the concerns raised about their quality and the reasons for their dismissal. Moreover, the Commission was informed that the concerns identified in the EFSA Conclusion (2017) were independent of the recognition of the studies submitted: even if the studies had been considered valid, the concerns could be considered even more robustly substantiated. In the EFSA Conclusion, 2017 (p. 10) it is explained that "a number of statements presented in the old dossier" are considered "as unreliable". This reflected the conclusion reached during the peer review, considering that the results of these studies could not be used for the risk assessment of *P. chlororaphis* MA342. The divergent opinions were noted in the Peer Review Meeting Report with RMS and three Member States' experts supporting the use of those study reports as supportive in a weight of evidence approach. EFSA also noted in the Conclusion the disagreement of the RMS with respect to some of the data gaps identified. However, no reference was made by EFSA to the conclusions derived in the earlier opinion of the Scientific Committee of Plants.

Terms of Reference

The potential for transport of DDR metabolite to plant parts following seed treatment, play a crucial role in the risk assessment and was not discussed in detail during the peer review of the renewal process. In particular, the translocation potential of *P. chlororaphis* MA342 was not discussed in detail.

With a view to the above background and in accordance with Art. 29 of Regulation (EC) No 178/2002 the Commission asks EFSA for an assessment on the capacity of *P. chlororaphis* strain MA342 to translocate to parts of plants after seed treatment and to proliferate, and to consider if it is possible to complete and/or refine the assessment of the exposure of consumers, workers and residents to

[1] "(…) the Committee concluded that the issue of residues has been adequately addressed that there is no cause for concern". Additionally, the Committee considered "the potential for human exposure to DDR as well as to other possible antibiotic metabolites so low that, even in the absence of further information, no major concern exists for consumer and operator safety". "*Pseudomonas chlororaphis* MA342 applied to seeds does not continue to colonise the emerging plant" and "in the absence of sustained colonisation, the number of *P. chlororaphis* associated with the harvested grain as well as the concentration of any metabolites produced would be very low" (European Commission, 2004).

metabolites produced by this microorganism following its use for seed treatment of cereals and peas using the information available in the dossier.

Further consideration on the aneugenicity of metabolite DDR may be required and addressed by EFSA.

For the assessment EFSA should consider all evidence available in the dossier for renewal of the approval. Clarity on both issues would facilitate the decision on the renewal of approval for *P. chlororaphis* MA342 and subsequently the decision on risk management measures for residues in food.

All the available information submitted during the evaluation of *P. chlororaphis* MA342 was accessed by EFSA.

1.2. Interpretation of the Terms of Reference

The European Food Safety Authority (EFSA) PPR Panel will develop a statement with a scientific advice on the translocation potential by *P. chlororaphis* MA342 in plants after seed treatment of cereals and peas and, if applicable, for a revision of the assessment of the risk for humans. No request has been expressed in the mandate to address uses on seed treatment of carrots and foliar treatment of cereals.

The PPR Panel interpreted that the terms of reference require the Panel to provide advice on:

- Translocation of the microorganism to edible parts of the plants and production of DDR there.
- Uptake of the metabolite DDR from soil and transport to the edible parts of the plants.
- Aneugenicity of metabolite DDR.
- Possible refinement of risk assessment for consumers, workers and residents to metabolites of *P. chlororaphis* MA342.

Since the statement is required with respect to a specific strain (MA342) of *P. chlororaphis*, all information in the dossier with respect to the identity and characterisation of this specific strain will be examined and considered by the PPR Panel.

Studies found in the applicant's dossier addressing the issues relevant to the required questions will be considered as background documents for this statement.

The PPR Panel accepted the mandate (European Commission, 2020) in relation to the conflicting appraisal of studies in the dossier. In particular, the different consideration of some studies by EFSA and the RMS and Co-RMS (in agreement with previous evaluation performed by the Scientific Committee of Plants (European Commission, 2002)).

The PPR Panel will consider the quality of studies presented by the applicant, since a number of the studies in the dossier that are not publicly available (not peer reviewed) have not been performed under GLP and issues in relation to their reliability were the origin of the discrepancies among the different evaluators. As regulation (European Commission, 2009, 2013) already foresees a derogation of the requirement of GLP status for studies supporting microorganisms, the PPR Panel may provide recommendations on minimum reporting and quality criteria that such studies should meet.

The PPR Panel noted that the species *P. chlororaphis* was discussed in an opinion of the EFSA Panel on Biological Hazards (EFSA BIOHAZ Panel, 2015) and it was not considered to be included in the list of microorganisms with a Qualified Presumption of Safety (QPS) status. The PPR Panel WG agreed to ask for a possible confirmation/update on the status of *P. chlororaphis* with respect to QPS criteria. As a result of this request, the EFSA Panel on Biological Hazards published the QPS opinion (EFSA BIOHAZ Panel, 2020) confirming that this species does not qualify as QPS due to a lack of the body of knowledge.

The PPR Panel also decided to consult the Standing working group on genotoxicity of the EFSA Scientific Committee for questions on aneugenicity of the metabolite DDR since a Guidance on aneugenicity assessment is currently being developed, and this is in a late stage of process.

2. Data and methodologies

2.1. Data

The PPR Panel based its assessment on documents and information contained in the dossier submitted to EFSA during the peer review of the risk assessment of the active substance *Pseudomonas chlororaphis* MA342.

The Panel also consulted the Opinion of the Scientific Committee on Plants on Specific Questions from the Commission, Regarding the Evaluation of *Pseudomonas chlororaphis* in the Context of Council

Directive 91/414/EEC (2002) (European Commission, 2002), the Renewal Assessment Report (RAR) prepared by the Netherlands as rapporteur Member State (RMS) (The Netherlands, 2016), the EFSA conclusion on the peer review of *P. chlororaphis* MA342 (EFSA, 2017) and the background documents to this peer review. All citations on studies and other documents can be found in the section References.

2.2. Methodologies

Studies in the dossier were examined and appraised for reliability on the basis of expert knowledge and according to the state of the art in the relevant fields (microbiology, chemistry/residues and genotoxicity). For transparency and easy comparison, these studies are quoted in the PPR Panel statement in the same way they are quoted in the RMS RAR. All the relevant studies in the dossier were examined in detail. Critical criteria considered in the assessment of each study are described together with the assessment. In one of the meetings of the working group, the PPR Panel invited a hearing expert nominated by the RMS to confirm the identification of the relevant studies in the dossier and to further elucidate the interpretation of the results of these studies.

As regards a possible reference point for aneugenicity of the metabolite DDR, the Standing working group on genotoxicity of the EFSA Scientific Committee was consulted, since the Draft Guidance on aneugenicity assessment is in a very late stage of development. During the peer review (see background documents to the EFSA Conclusion, EFSA, 2017), there were discussions if the most sensitive endpoint for aneugenicity for DDR was appropriately assessed and the final conclusion was different from the conclusion of the Scientific Committee on Plants (European Commission, 2002). Therefore, the PPR Panel has asked the Standing working group on genotoxicity for advice on the two available studies for aneugenicity, to allow the PPR Panel to derive a conclusion on the following contradictory discussions from the peer review:

a) Can a NOEL for aneugenicity be reliably set based on the *in vivo* MN assay with DDR (Abramsson-Zetterberg, 2000)?
b) If a NOEL for DDR can be reliably set in the *in vivo* MN assay (Abramsson-Zetterberg, 2000), is this based upon non-disjunction or chromosome loss?
c) Has the most sensitive endpoint for aneugenicity in the *in vivo* MN assay with DDR (Abramsson-Zetterberg, 2000) been reliably investigated?

Regulation (European Commission, 2009, 2011, 2013) and risk assessment guidance documents (European Commission, 2012, 2017), as well as OECD Guidance (OECD, 2016) relevant to this statement were considered by the PPR Panel.

3. Assessment

3.1. Taxonomic identification

Assessment of data in the applicant's dossier

No issues in relation to the identity of the microorganism were identified during the peer review and considered in the EFSA's conclusion (EFSA, 2017) and its examination is not directly required for this mandate. Since the applicant proposed in the dossier to read across and bridge information of other strains of *P. chlororaphis* and other *Pseudomonas* species, information in relation to the taxonomic identification of strain MA342 as a *P. chlororaphis* is considered crucial to appraise the robustness and/or uncertainties associated with the bridging of information across strains and/or species.

The following information is found in the dossier in relation to the identification and taxonomic classification of *P. chlororaphis* MA342.

The microorganism *P. chlororaphis* strain MA342 has been deposited in 1994 in the National collection of industrial food and marine bacteria (NCIMB), Sweden, under the deposition number NCIMB 40616 (NCIMB, 1994).

A short history of the taxonomic insights in the *P. chlororaphis* species up to 1989 is presented in an undated report (Department of Plant Pathology and Biological Control, date unknown).

Identification of the isolate was based on fatty acid analysis (Anonymous, 1993a). For this only four strains were used for comparison, one *P. chlororaphis* strain and three strains of *P. fluorescens.* Analysis showed the most homology with the *P. chlororaphis* strain. Due to the limited number of strains used for this analysis, the confidence in the identification outcome is considered low.

Laguerre et al. (1994) reported the identification potential of Restriction Fragment Length Polymorphism (RFLP) analysis of the 16S rRNA gene for several species of *Pseudomonas spp*. Because of the limited number of strains tested, uncertainty remains on the wider applicability of the technique for species identification within the *Pseudomonas* genus.

Moore et al. (1996) compared the 16S rRNA sequence of 21 species belonging to the *Pseudomonas* genus, including two *P. chlororaphis* strains and found that this sequence shared 99.5% identity with *P. aureofaciens* strains and 98.5% with *P. tolaasii* strains. However, this study did not include *P. protegens,* closely related to *P. chlororaphis* (Ramette et al., 2011). Therefore, it is not clear if the similarity of only the 16S rRNA sequence is sufficient to identify *P. chlororaphis* to the species level.

The RFLP analysis of the 16S rRNA gene and randomly amplified polymorphic DNA (RAPD) showed differentiation of the *P. chlororaphis* MA342 strain from 11 *P. chlororaphis* reference strains (Anonymous, 1997a). The fact that the reference strains could be differentiated in several different 16S rRNA RFLP types was unexpected based on the reported results of Moore et al. (1996) who showed a high degree of identity of the 16S rRNA sequences among the *P. chlororaphis* strains and its closely related *Pseudomonas spp.* This raises doubts on the correct identification of the reference strains used in this study.

Discussion of the data from the applicant's dossier in relation to the state of the art

The taxonomic knowledge on *P. chlororaphis* has been established in several studies published in peer-reviewed scientific literature. Within the *P. chlororaphis* species, several subspecies (subsp. *chlororaphis,* subsp. *aureofaciens,* subsp. *aurantiaca* (Peix et al., 2007) and subsp. *piscium* (Burr et al., 2010) were identified. *P. chlororaphis* is closely related to *P. protegens* which was also isolated from the plant rhizosphere (Ramette et al., 2011). As a consequence, the method used for the identification of *P. chlororaphis* MA 342 would need to take this knowledge into account and be able to differentiate the strain from its closest relatives.

Uggla (2000) quoted a personal communication with Borowicz on production of DDR by soil rhizosphere bacteria, without giving more details. The PPR Panel retrieved the Borowicz Doctoral Thesis, which was consulted for the information referred in Uggla (2000). Borowicz (1998) doctoral thesis suggested that *P. chlororaphis* MA342 should be reclassified on the basis of 16S rRNA gene sequence analysis to a new species named *P. borealis*. This species was, however, not validly published, and therefore, *P. borealis* does not belong to the list of names with Standing in Nomenclature (Parte et al., 2020). The name was used in relation to *Pseudomonas* strains with ice nucleation potential (Wilson et al., 2006; Wu et al., 2009). The 16S rRNA gene sequence analysis showed clearly the distinct phylogenetic position of the strain MA342 from the *P. chlororaphis* type strain LMG 5004 (Borowicz, 1998).

Conclusion

There are no data available in the dossier confirming the identity of the strain designed as *P. chlororaphis* MA342 as belonging to the species *P. chlororaphis*. The microorganism was characterised according the methodology available at the time of the first authorisation, i.e. before 2001. No updated identification according the state of the art is available in the renewal dossier. This is a source of uncertainty in relation to the relevance of data of papers referring to only the species, not mentioning the specific strain MA342. The results of Borowicz (1998), based on the 16S rRNA sequence analysis, indicate that the strain MA342 is most probably not belonging to the species *P. chlororaphis*. As a consequence, studies provided in the dossier not related to strain MA342, but to other strains of the species, *P. chlororaphis* should be taken into account with care due to the high uncertainty of their relatedness with strain MA342.

3.2. Translocation of the microorganism and production, uptake and degradation of metabolite DDR

3.2.1. Microorganism translocation and internalisation

Assessment of data in the applicant's dossier

The studies in the dossier containing information or investigating the translocation and proliferation of the microorganism in plants were identified and considered by the PPR Panel. The assessment of the studies examined is presented below.

Bennett and Whipps (2008) described an investigation where, after application of *P. chlororaphis* MA342 to seeds during drum priming, it was isolated from seeds, roots and rhizosphere soil, with a declining in number over the tested time frame of 8 weeks.

Anonymous (1997a,b) described a field experiment in which seeds were inoculated with wild-type *P. chlororaphis* MA342 and with a rifampicin resistant variant of the strain. After shooting, the rifampicin resistant *P. chlororaphis* MA342 was re-isolated from the soil but not from the roots and the shoots. However, no evidence was provided that the plants were colonised by the strain and beneficial effects on the plants were not reported. Therefore, uncertainty remains on how far this experiment is representative for the real practical application of the biocontrol agent.

Anonymous (1993a,b, 1997a,b, 1998b) also used a rifampicin-resistant variant of *P. chlororaphis* MA342 to treat seeds, which were sown in unsterilised soil in pots and followed for 27 days. *P. chlororaphis* MA342 was isolated after 27 days from roots (2.10^2 cfu/g), leaf 1 (20 cfu/g), leaf 2 (2 cfu/g), leaf 3 (0.2 cfu/g) and leaf 4 (0 cfu/g). These experiments support the translocation of *P. chlororaphis* MA342 from the seeds to roots and plant tissue, although at low concentrations.

Anonymous (1998b) used a *P. chlororaphis* MA342 strain marked with a *gfp* gene (encoding the green fluorescent protein) and containing a kanamycin-resistant gene. The treated seeds were grown in tubes with unsterilised soil. *P. chlororaphis* colonies were found back after 9 weeks on seeds, coleoptile and seminal roots. The presence of MA342 bacteria was also tested in leaf sheets at 9 weeks. They were found back in the phyllosphere (estimated concentration based on Figure 1 in the report of about 10^3 cfu/plant), which is a lower concentration compared to the rhizosphere (about 10^6 cfu/plant, expressed in the paper per plant without details on how it was exactly measured). This experiment also supports the possibility of translocation of *P. chlororaphis* MA342 from the seeds to roots and plant tissue.

Tombolini et al. (1999) investigated the pattern of colonisation and cell aggregation of *P. chlororaphis* MA342 in barley seeds before and after sowing in tubes in a greenhouse experiment. The experiment lasted for 14 days and only the embryo was tested. They used a strain tagged with the *gfp* gene (encoding the green fluorescent protein). After sowing, they showed an irregular distribution of bacterial aggregates reflecting epiphytic colonisation of glume cells. There was a trend of aggregation near the embryo, but never within the embryo. The discussion section of Anonymous (1998) quoted a personal communication of Tombolini to highlight the poor colonisation that occurs in greenhouse experiments, which implies that these experiments may not be representative for field use.

McInroy and Kloepper (1995) isolated *P. chlororaphis* strains from the roots and the stem of *Zea mays* and *Gossypium hirsutum* L. (cotton) in a field trial using untreated seeds. The strains were identified by fatty acid methyl-esters (FAME) analysis. Since this study did not report results of strain MA342, uncertainty remains on its relevance.

Snyder et al. (1998) showed that the *P. chlororaphis* strain L11 was able to invade the roots and move from the roots to the foliage of corn. They found concentrations of the strain (expressed in cfu/g wet weight plant tissue) in the range of 10^7–$10^{8.7}$ in roots and of $10^{2.8}$–10^5 in the stem. No other plant parts were tested. This study shows that translocation occurred from the roots to the stem in corn, although strain MA342 was not investigated in this study. Thus, uncertainty remains on its relevance.

The paper of Liddell and Parke (1989) described the root colonisation by a strain of *P. fluorescens* after coating of pea seeds. The density of *P. fluorescens* and the depth of the roots where it was detected depended on the water transport in the soil. This study is considered as not relevant for the question asked.

The paper of Yan et al. (2003) investigated colonisation of tomato roots by *P. fluorescens*. No data on *P. chlororaphis* were presented. Therefore, the study was not considered relevant for this assessment.

Discussion of the data from the dossier in relation to the state of the art

The data presented in the dossier provide already an indication on the potential of translocation of *P. chlororaphis* strain MA342 to other plant tissues (Anonymous, 1993a,b, 1998b). These data also indicate that the concentrations reached in the plant foliage were much lower (at least 100 times lower) than those found within the root tissues.

Scientific peer-reviewed literature about soil bacteria adds further evidence to the possibility of translocation of *P. chlororaphis* MA342 from treated seeds to other plant tissues and provides also indications that these bacteria get internalised in the plant tissues. By seedborne applications, the plant will be colonised with the biocontrol agent to perform its biocontrol functions (Finkel et al., 2017,

Mercado-Blanco and Bakker, 2007). This colonisation implies growth of *P. chlororaphis* in the rhizosphere and in the plant parts grown above the ground.

Plant growth-promoting rhizobacteria (PGPR), including *Pseudomonas* species used for biocontrol, are recognised in several studies to be able to penetrate into the roots establishing an endophytic relation, supported by microscopic proof and their ability to re-infect surface disinfected seeds (for review, see Mercado-Blanco and Bakker, 2007). PGPR may enter and establish, as root endophytes through root hair cells, root cracks and tissue wounds occurring during plant growth. Also plant parts above the ground as leaves have attachment points on the surface of the plant and entry points to get bacteria translocated internally in the plant. PGPR *Pseudomonas spp.* were isolated as endophytic organisms in several plant organs from several plant species (e.g. Reiter and Sessitsch, 2006 who detected endophytic *Pseudomonas* spp. from leaves and flowers from *Crocus* plants in a natural habitat by polymerase chain reaction (PCR) detection). Reiter and Sessitsch (2006) noticed a difference in the culturable component of the microflora compared to the composition determined by molecular, non-culturable methods. The authors recommended a combination of detection techniques for endophytic microorganisms based on culture and non-culture methods.

Andreoli et al. (2019) investigated endophytic behaviour of *P. protegens* MP12, a strain which is used as a biocontrol strain for grapevine, using an *in vitro* system of root inoculation. Endophytic presence was detected in 42–65% of the plants colonised with 4.22 till 4.65 log CFU/g from day 25 till day 125 after inoculation. The roots were colonised with 3.57 log CFU/g, the lower shoots with 5.91 log CFU/g, the higher shoots with 4.36 log CFU/g. Sun et al. (2014) found similar concentrations on ryegrass roots and shoots after inoculation of roots for 3–15 days with *Pseudomonas sp* Ph6 (5.51–5.79 log CFU/g in roots and 3.65–4.60 log CFU/g in shoots).

Literature on human pathogenic *E. coli* and *Salmonella* demonstrated the occurrence of internalisation of these bacteria from contaminated soil to plant roots and translocation to plant leaves (Van linden et al., 2013, Jechalke et al., 2019, Kljujev et al., 2018). Concentrations up to 4.41–5.92 log CFU were found per seedling (Van der linden et al., 2013). Colonisation rates were affected by soil type, plant species and strain (Jechalke et al., 2019). Bacteria were also found in the plant cells cytoplasm, although the mechanism of their entrance has not been clarified yet (Kljujev et al., 2018).

Conclusion

Based on the studies in the dossier supported by literature data, it can be assumed that *P. chlororaphis* MA342 used as a biocontrol agent by seedborne application, could be present internally in the edible plant tissues. Based on these data, the internalised bacteria can survive, move in the plant and grow till an estimated concentration up to about 10^5 cfu/g (Snyder et al., 1998; Andreoli et al., 2019). As *P. chlororaphis* is not recommended for the QPS status and uncertainty remains on the taxonomic identity of the strain MA342, a risk assessment of the presence of *P. chlororaphis* strain MA342 in edible plant tissues would be necessary.

It is not possible by simply washing edible plant parts to remove attached or internalised bacterial cells. Also, no industrial systems are available or currently in use for decontamination of the plants sufficiently to remove or kill all bacterial cells on freshly consumed plant commodities. Therefore, some exposure to *P. chlororaphis* MA342 has to be assumed by consumption of fresh commodities. Nevertheless, the processing of these raw commodities with methods involving sufficient heating (e.g. by cooking or baking processes for sufficient time/temperature combination) will result in the death of the bacteria.

3.2.2. Metabolite DDR

Production of secondary metabolites by P. chlororaphis MA342

The biological effect of *P. chlororaphis* MA342 as biocontrol agent against *D. teres (Drechslera teres)* and other fungi was demonstrated to be caused by the secondary metabolites excreted by the microorganism (Department of Plant Pathology and Biological control, 1997, 1998). In the former report, it was shown that the crude supernatant fraction (in the absence of the microorganism) was almost as effective as the bacterium and that the bacterium is mainly active through antifungal metabolites. In the latter report, an experiment is described where MA342 was grown in TSB (15 g Tryptic Soy Broth in 1 L distilled water) at 20°C for 48 h. The supernatant was collected and fractioned by different means (no details given) into nine different fractions which were concentrated and tested for biocontrol against *D. teres* in a greenhouse bioassay. Different levels of biocontrol effects were observed for seven out of the nine fractions. The authors hypothesise that there could be at least five

different metabolites involved in the biocontrol effect of strain MA342. Individual metabolites were not identified in this paper, but it shows how secondary metabolites can be produced by *P. chlororaphis* MA342 when it is grown under laboratory conditions.

From the information provided in the reports, it was not possible to confirm or exclude the formation of metabolites under naturally occurring conditions in soil and plants. Nevertheless, the reports show that the presence of fungi or stressed environmental conditions are not necessary for *P. chlororaphis* MA342 to produce and excrete the bioactive secondary metabolites.

Production of DDR in plants

According to RMS and co-RMS (The Netherlands, 2016, The Netherlands and Denmark, 2017), DDR is only produced during the growth stage of *P. chlororaphis* MA342 and when fungi are present, but no supporting evidence has been found in the applicant's dossier. The RMS hearing expert confirmed that this statement was not supported by any specific study in the dossier but was rather an assumption based on general biological properties of the microorganisms and of the function and mechanism of action of this kind of secondary metabolites. There is no specific study in the dossier on DDR production during different growth stages. Also, there is no evidence that DDR is not produced during the stationary phase of the culture. Other strains of *P. chlororaphis* are known to produce biologically active secondary metabolites (as phenazine-1-carboxamide, PCN) not during the growth phase but during the stationary one (see e.g. van Rij et al., 2004). In this case, the microorganisms produced secondary metabolites during the stationary phase and not during the logarithmic phase.

There are no studies in the dossier investigating the production of DDR in the presence or absence of the fungi. However, there are evidences that may question the assumption that the metabolite is only produced when a pathogen is present, since the metabolite is found in the production batches, where pathogenic fungi is not expected to be present. In addition, studies discussed above (Department of Plant Pathology and Biological control, 1997, 1998) indicate that *P. chlororaphis* MA342 may produce metabolites in the absence of the fungi or any particularly stressed condition.

The available data show that MA342 may grow within the plant which implicates that DDR may be produced (see Section 3.2.1). Growth of *P. chlororaphis* MA342 to concentrations up to 10^5 cfu/g in upper plant parts is relatively low compared to its optimum growth rate (10^8–10^9 cfu/g) and it can be assumed that internally in the plant, the metabolic state of the bacteria is decreasing at a certain stage explaining this limited growth potential. A reduced activity of the growth and metabolic activity of the bacteria is also supported by the fact that Anonymous (1993a,b, 1997a,b, 1998b) only detected a very low amount of internalised *P. chlororaphis* MA342 concentration in edible plant parts by culturing. Reiter and Sessitsch (2006) recognised a higher amount of *Pseudomonas* bacteria by molecular culture independent methods compared to methods based on culturing. They used molecular methods detecting all bacterial cells, also those which are no longer metabolically active. Therefore, a significant growth and metabolic activity of *P. chlororaphis* MA342 within the plants are not expected. However, a decrease in active growth would not necessarily lead to a decrease in production of DDR as a secondary metabolite.

The RMS considered the production of the metabolite within the plant unlikely since DDR is also phytotoxic and if it would be produced, then damage to the plants will be observed. However, this circumstantial evidence cannot be confirmed since studies showing DDR phytotoxicity are not included in the dossier.

Therefore, with the available information in the dossier, it is uncertain if lower levels of DDR would be expected in the plant parts and seeds compared to the soil. In conclusion, it cannot be excluded that at least some DDR can be produced by the microorganism when translocated to the plant.

Production of DDR in soil

There is no specific study in the dossier investigating the production of the metabolite in soil. The RAR stated that the metabolite DDR is also produced by other microorganisms (by up to 2% of soil rhizosphere microorganisms according to a personal communication quoted in Uggla (2000)). The PPR Panel traced the origin of this statement to the Doctoral thesis by Borowicz (1998) and could only confirm that other strains also produce this metabolite, but no information on the actual levels was reported. Therefore, it was concluded that there is no information in the dossier to verify the statement in relation to the amount of soil bacteria producing this metabolite.

Background levels of naturally occurring substances are one aspect that the regulation (European Commission, 2009, 2011, 2013) considers for this kind of substances. The amount added by the active substance to naturally occurring levels would then need to be considered in the risk assessment. The

PPR Panel recognises the difficulty to estimate background levels. During the meeting with the hearing expert, it was confirmed that there is no information on background levels of DDR or any other metabolites produced by *P. chlororaphis* MA342. However, it is generally known that rhizoxins are produced by other species and it cannot be excluded that this is the case also for DDR. This constitutes an additional uncertainty to consider for the risk assessment. In addition, for the application of the TTC approach, all sources of exposure to the same metabolite would need to be considered for a reliable consumer risk assessment.

Additionally, there is no information in the dossier investigating the uptake of DDR by plants from soils and its transport from roots to other plant parts.

Therefore, with the information available in the dossier, it is not possible to confirm or exclude the possibility of residues of DDR in plant commodities coming from its occurrence/production in soil.

Degradation of DDR

A study on degradation of DDR (Eriksson, 2000a, B.2.1.8) was included in the dossier and indicated a rather short half-life (125–1,505 min). However, this was a study on hydrolysis in a bacterial suspension as a function of pH, claimed to be performed according to OECD guideline 111. The study had some shortcomings, among other things that it was not a good laboratory practice (GLP) study and hydrolysis of DDR was tested at the temperatures 8 and 20°C and pH 2 and 9, which are extreme values compared to pH values normally found in the environment and which are recommended to be tested (pH 4, 7 and 9). These limitations were also identified during the peer review (EFSA, 2017). Additionally, the purpose of this OECD test is to assess abiotic hydrolytic transformations of chemicals in aquatic environments, and it could be questioned if this test is relevant for the degradation of DDR in the soil and the representative crops (cereals and peas).

It is possible, even when secondary metabolites as DDR would be produced during certain stages of the plant growth, that these, depending on their stability, would get degraded between harvest and consumption. Further processing of edible plant parts before consumption may also enhance degradation of secondary metabolites as DDR.

However, no relevant data (e.g. metabolism and processing studies) are available to confirm these assumptions and to do a quantitative estimation of the extent of the potential degradation of DDR.

Residues in plants

Regardless of whether the metabolite is formed in soil and transported, or formed directly in the plant, without subsequent transformation leading to degradation of DDR, exposure of the consumers will be driven by the levels of DDR in the plant commodity.

In the dossier, there were no proper field residue trials, performed according to guidelines, investigating the levels of non-viable and viable residues after application of *P. chlororaphis* MA342 according to the supported good agricultural practice (GAP). There were, however, some studies available measuring the levels of DDR in plant parts grown from treated barley seeds. After sowing the seeds in soil, the pots were kept for 10 days at 6°C for germination, and were then moved to the greenhouse, at 20°C, and kept there for an additional 12 days. None of these studies were performed according to any specified guideline or GLP, and the reporting was limited. In one of the studies, Eriksson (2000b), plants were harvested at BBCH 13 (about 22 days after sowing, at three leaves stage) and roots and kernels were sampled and analysed, with DDR levels being < 1 and < 3 μg/kg, respectively. The author considered that the analytical method of this study needs to be improved, as the chlorophyll content causes great disturbance. The experiment was carried out in a greenhouse and no information on the degree of colonisation of the plants was provided. In another study (Eriksson, 2000c), plants from untreated and treated barley seeds (four replicate samples for each treatment) were harvested at BBCH 13 (three leaves stage) and shoots were sampled and analysed. No detectable amounts of DDR were found, the levels in all samples were below the limit of quantification (LOQ) (1 μg/kg). However, the plant material was derived from a greenhouse experiment and no data were included on whether the treated plants were colonised by *P. chlororaphis* strain MA342. In Eriksson (2000d), DDR was analysed in two samples of grains harvested from plants grown from treated barley seeds and no detectable amounts of DDR were found (< 1 μg/kg). However, this study was the analytical report, without information regarding when the grains were harvested, the amount of the sample etc. No information was provided if samples were from a greenhouse experiment or a field trial. Therefore, the results are considered not reliable.

In relation to the greenhouse experiments, Hökeberg et al., 2000 stated the following observation: 'Poor root colonization was observed on seedlings grown at both 6 and 14°C, and also in pilot

experiments in which barley seeds were grown in greenhouse at 20°C'. This citation was also found back in Anonymous (1998b) as a personal communication from Tombolini. It could therefore be questioned if *P. chlororaphis* MA342 in fact had colonised the roots of the plants in these experiments, and if there was a potential for DDR production. No check or confirmation was included in the studies regarding if the treated plants were colonised by *P. chlororaphis* strain MA342.

It is therefore concluded that the reliability of these studies considering DDR production is low. Therefore, uncertainty remains regarding the amount of DDR produced in plant material derived from seeds treated with *P. chlororaphis* MA342 and the possible degradation of DDR in plant parts and the resulting levels in the plant commodities.

In a more recent study (Aversa, 2015), levels of DDR in wheat seeds harvested from plants treated with *P. chlororaphis* strain MA342 were determined. This was a GLP study, however, the focus was to validate the analytical method used, and detailed information regarding the field trial part of the efficacy test (treatment, application rate and type, time of sampling etc.) was not included in the applicant's dossier. In this report, only two samples were analysed, from two efficacy trials with wheat treated with Cerall (Plant protection product containing *P. chlororaphis* MA342), one from Sweden and the other from Denmark. The content of DDR in these samples was < LOQ (1 μg/kg). According to the RMS, who got access to the efficacy trial after the peer review, the wheat plants were treated with foliar application. Since this study was not available in the dossier, the reliability could not be properly evaluated by the PPR Panel and it is uncertain if these results are relevant also to the seed treatment considered in this statement.

The PPR Panel also noted that there are no data available in the dossier on the residue levels of DDR in peas at harvest after seed treatment according to the proposed GAP. Peas belong to another crop group and according to the EU guidance document on data requirements for setting maximum residue levels (MRLs) (European Commission, 2005, 2017), it is not possible to extrapolate residue data for cereals to the groups of legumes or pulses (it is not clear which type of peas is included in the intended use). It is possible that the behaviour of the microorganism differs depending on the host plant. It is therefore the opinion of the Panel that information on translocation of *P. chlororaphis* strain MA342, as well as residue levels of DDR in peas is needed, or a scientific justification why this would not be considered necessary.

Conclusion

There is limited or no information in the dossier regarding the production of DDR by *P. chlororaphis MA342* in the soil, during different growth stages, in the presence/absence of pathogens or in plants grown in the field from treated seeds. Additionally, there are no data on background levels of DDR in the soil and possible uptake of DDR by plants from soil. A hydrolysis study performed at extreme pH conditions provided indications that DDR is degraded rather rapidly, but there are no reliable data available to confirm that this is the case under environmentally realistic conditions and in the treated crops. Likewise, studies analysing the content of DDR in different plant parts grown from treated cereal seeds showed that the levels were below the level of quantification (< 1–3 μg/kg). The reliability of these studies is, however, questioned, since there was no confirmation that the plants used in these studies were properly colonised by *P. chlororaphis* MA342. In this case, no DDR would even have been produced in the plants. For peas grown from treated seeds, there are no studies or information about translocation of *P. chlororaphis* MA342 or transport of residues of DDR.

It could be assumed that processing of the raw agricultural commodities before consumption (which is always the case at least for cereals) may enhance degradation of secondary metabolites as DDR. Thus, performing the consumer exposure assessment using levels at the LOQ is most probably an overestimation, but the information available does not allow to conclude on a specific lower level to be used. In addition, no information on the reaction, transformation or degradation products of DDR is provided in the dossier.

The overall conclusion is that DDR is likely present in the edible crops only at low levels, but with the data available in the dossier, it is not possible to conclude at which specific level. Thus, the exposure level assumed in the EFSA conclusion (EFSA, 2017) for the consumer risk assessment (based on LOQ = 1 μg/kg) cannot be refined unless a fast degradation is demonstrated in realistic scenarios and detailed residue trials determining the residue levels of DDR (preferable with an analytical method with a lower LOQ) following application treatments according to the supported GAPs are provided.

3.3. Genotoxicity of metabolite DDR

3.3.1. Aneugenicity

In 2002, the Scientific Committee on Plants produced an opinion (European Commission, 2002) on specific questions regarding the evaluation of *P. chlororaphis* MA342. Among others, one of the concerns of the European Commission was if the toxicological safety of the antibiotic metabolites of *P. chlororaphis* MA342 has been adequately addressed.

The Scientific Committee on Plants concluded that the information on toxicity of *P. chlororaphis* MA342 is limited, because

- no toxicokinetic data on DDR are available (absorption, biotransformation, distribution),
- only two studies on the genotoxicity of the metabolite DDR have been carried out (an *in vitro* (V79 Chinese hamster cells), and an *in vivo* MN assay in mice by gavage), and showed chromosomal damage,
- no experiments have been performed to evaluate the mutagenic potential of DDR.

Despite the limited data package, the Scientific Committee on Plants concluded that even in the absence of further information, no major concern existed for consumer and operator safety. The Scientific Committee on Plants justified this conclusion based upon a low potential of exposure to *P. chlororaphis*. As regards the *in vivo* MN assay (Abramsson-Zetterberg, 2000), the Scientific Committee on Plants concluded that an NOEL for a transient increase in micronucleated polychromatic erythrocyte was set at 0.2 mg/kg body weight (bw), the lowest dose tested.

During the renewal of *P. chlororaphis* MA342, again the endpoint for aneugenicity was discussed. In the initial review assessment report (The Netherlands, 2016), the RMS proposed the NOEL for aneugenicity in the *in vivo* MN assay (Abramsson-Zetterberg, 2000) at 2 mg/kg bw, the mid-dose tested. In the peer review (EFSA, 2017), experts agreed that considering results from an *in vitro* genotoxicity study (Önfelt et al., 2000; non-GLP and not guideline compliant), DDR is assumed to be a specific and efficient microtubule inhibitor. As regards the NOEL proposed in the micronucleus assay, the experts concluded that there was uncertainty about this since an aneugenic effect due to non-disjunction (as shown by Önfelt et al., 2000) can be induced at lower concentrations than an aneugenic effect by chromosome loss. In the absence of further data, the experts agreed to apply the TTC threshold for genotoxic compounds. This resulted in the conclusion that there was no safe use for consumers as regards exposure to DDR, even if only the LOQ is used as exposure value.

Within the current mandate, the Standing working group on genotoxicity of the Scientific Committee followed the PPR Panel request for advice on the available aneugenicity studies. The advice, formulated as re-assessment of the two available studies, is included as original statement in the Annex A to this Scientific Statement.

The working group on *P. chlororaphis* MA342 of the PPR Panel considered the advice and interpreted the results in the light of discussions from the peer review.

The *in vitro* study (Önfelt et al., 2000) had an appropriate study design to investigate the analysis of mitotic abnormalities, but no data were reported, and no results adequately presented in the study report. Neither the number of tested doses nor the exact tested concentrations were reported. The authors stated that a statistically significant increase of c-mitosis was observed at 2.5×10^{-11} M while at 5×10^{-11} M, no statistically significant increase was observed compared to control value. As no evaluation of these data was reported in the study, the submitted version of the study cannot be taken into account as reliable to conclude that DDR is an inhibitor of mitosis *in vitro*.

The *in vivo* study (Abramsson-Zetterberg, 2000) was meant to be the follow-up of the *in vitro* study. Since inhibition of mitosis was assumed by the authors to be the proven mechanism, based upon the *in vitro* study (Önfelt et al., 2000), no kinetochore staining or fluorescence in situ hybridisation (FISH) with pan-centromeric probes was done in the *in vivo* MN assay. Three doses (0.2, 2.05 and 18.6 mg/kg bw) were tested. A statistically significant increase of MN was observed only at the highest dose tested at 38 h sampling time but not at 62 h. Since only few doses were tested in the *in vivo* assay, together with lack of kinetochore staining in the *in vivo* MN assay (since results from the *in vitro* study are not taken into account), it is not possible to draw a reliable conclusion that DDR is producing an aneugenic effect nor to determine a threshold dose for aneugenicity.

DDR induces chromosomal damage; however, it was not evaluated if it occurred through aneugenic or clastogenic mechanism. Therefore, no reference values for DDR, based upon aneugenicity endpoint, can be derived in order to refine the consumer risk assessment.

The Standing working group on genotoxicity of the EFSA Scientific Committee and the PPR Panel working group on *P. chlororaphis* MA342 made the evaluation and drew the conclusion in alignment with the Draft Guidance on aneugenicity assessment (2020), which is in a very late stage of development.

3.3.2. Other genotoxic endpoints

No other studies addressing the genotoxicity of DDR were provided either in the original dossier, or for the renewal of *P. chlororaphis* MA342. Hence, no conclusion on e.g. gene mutation as genotoxic effect can be made. Since DDR contains an epoxide functional group, gene mutation potential cannot be excluded.

3.3.3. Other consideration for the risk assessment

As explained in the EFSA Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA Scientific Committee, 2019), when structural alerts or chemical-specific genotoxicity data, such as Ames test results, indicate that the chemical has the potential to be a DNA-reactive mutagenic and/or genotoxic carcinogenic substance, a TTC value of 0.0000025 mg/kg bw per day can be applied. For reliable application of the TTC approach for pesticides residues (EFSA Scientific Committee, 2019), potential exposure to DDR from other possible sources (e.g. other soil living *Pseudomonas* species which can produce DDR) should be taken into account in order to ensure that total exposure to DDR is appropriately assessed.

4. Uncertainties

- **Study reporting and reliability**: a great number of key studies in the dossier are reports of research performed by the Swedish University of Agricultural Sciences (Uppsala, Sweden). Despite the studies were not performed under GLP or in an officially recognised laboratory in the sense established by the regulation (European Commission, 2013) and that they have not been published in a peer review journal, the results of these studies have been considered as generally reliable by the PPR Panel based on trust in the scientific excellence of the corresponding University. However, details in these reports are frequently scarce, above all in relation to the material and methods employed and raw results. In certain cases, the studies are outdated and with limited value in relation to recent knowledge (e.g. studies on taxonomic identification and translocation). Therefore, there is a certain grade of uncertainty with respect to the interpretation of the findings reported and their possible generalisation. In other instances, reports only provide a partial record of a wider investigation (e.g. analytical phase of a field trial). As the reports on the background studies (e.g. field trial) are not present in the dossier, it is difficult for the PPR Panel to draw definitive conclusions from these studies.
- **Taxonomic identification of strain MA342**: based on available information in the dossier, the PPR Panel considers that the strain MA342 is most probably not belonging to the species *P. chlororaphis*. Consequently, studies provided in the dossier not related to strain MA342, but to other strains of the species, *P. chlororaphis* should be considered with care due to the high uncertainty of their relatedness with strain MA342.
- **Background levels of DDR:** no information on background levels of DDR, or any other metabolites produced by *P. chlororaphis* MA342, is provided in the dossier. However, it cannot be excluded that DDR is also produced by other soil bacteria species. This constitutes an additional uncertainty to consider for the risk assessment. In addition, for the application of TTC approach, all sources of exposure to the same metabolite would need to be considered for a reliable consumer risk assessment.
- **Production, degradation uptake and transport of metabolites**: information on the production, degradation and possible uptake and transport from soil of the metabolite DDR is scarce and of limited relevance for normal environmental and field conditions. No guideline studies on degradation and soil or plant metabolism studies are available. No field residue trials on viable and non-viable residues are available. Possible transformation or degradation products of DDR are unknown. The limited information in these areas makes any estimation on

the levels of DDR in food commodities highly uncertain, compromising any possible refinement of the consumer's exposure to DDR. Additionally, information in the dossier shows that other metabolites are produced and play a role in the biological control, but there is no information about the levels of these or their (eco)toxicological relevance.

- **Absence of residue data for the use in peas**: information about the presence of *P. chlororaphis* MA342 and DDR or other potential secondary metabolites in peas grown from treated seeds is completely absent in the dossier. Most available information refers to uses in cereal crops. Therefore, extrapolation of any of the estimations and conclusions based on the dossier studies to the uses on peas is highly uncertain.
- **Aneugenicity**: The *in vitro* study (Önfelt et al., 2000), designed to analyse mitotic abnormalities, did not contain sufficient information in the report as submitted to reliably conclude that DDR is an inhibitor of mitosis *in vitro*. An *in vivo* MN assay (Abramsson-Zetterberg, 2000) was conducted to confirm an aneugenic potential of DDR, as hypothesised based on the *in vitro* study. However, as kinetochore staining was not performed and too few doses were tested, it was not possible to conclude on the aneugenic potential and set an aneugenicity reference point.

5. Conclusions

As regards the terms of reference as **translocation of the microorganism and transport of metabolite DDR to edible parts of the plants,** the PPR Panel concludes that:

There are no data available in the dossier confirming the identity of the strain designed as *P. chlororaphis* MA342 as belonging to the species *P. chlororaphis*. As a consequence, studies not related to strain MA342, but to other strains of the species, *P. chlororaphis* were considered with care due to the high uncertainty of their relatedness with strain MA342.

With the available information, it can be assumed that *P. chlororaphis* MA342, used as a biocontrol agent by seedborne application, could be present internally in the edible plant tissues. The internalised bacteria can survive, move in the plant and grow till an estimated concentration up to about 10^5 cfu/g.

Some exposure to *P. chlororaphis* MA342 can be assumed by consumption of fresh commodities. However, suitable processing (e.g. involving sufficient heating) of fresh commodities will result in the death of the bacteria.

The potential production of DDR by the microorganism within the plant cannot be excluded.

There is no information in the dossier regarding the production of DDR by *P. chlororaphis* MA342 during different growth stages. No information on DDR background levels or its production in soil and uptake and transport by plants is available. Therefore, it is not possible to confirm or exclude the possibility of residues of DDR in plant commodities coming from its occurrence/production in soil or the plant.

Whereas the PPR Panel considers that it is highly likely that DDR will degrade to a certain extent before consumption, no relevant data (e.g. metabolism and processing studies) are available in the dossier to do a quantitative estimation of the extent of this potential degradation.

The PPR Panel concluded that the reliability of the studies analysing the content of DDR in different plant parts grown from treated cereal seeds, which showed that the levels were below the level of quantification ($< 1–3$ μg/kg), is low. For peas grown from treated seeds, there are no studies or information about translocation of *P. chlororaphis* MA342 or transport of residues of DDR. In conclusion, the estimated levels of exposure based on the LOQ in the cereal samples analysed for DDR ($< 1–3$ μg/kg) cannot be further refined with the information available in the dossier.

As regards the terms of reference on **aneugenicity of metabolite DDR,** the PPR Panel concluded that:

Based on the results from the two available genotoxicity studies, DDR induces chromosomal damage. However, due to limitation in reporting and in study design, it is not possible to conclude if the damage occurred through aneugenic or clastogenic mechanism. Therefore, no toxicological reference values for DDR, based upon aneugenicity endpoint, can be derived in order to refine the consumer risk assessment.

Finally, since neither the exposure nor the toxicity endpoint can be refined with data available in the dossier, the PPR Panel cannot refine the risk assessment for the consumers or complete the risk assessment for workers and residents exposed to DDR. Thus, the concerns identified in the EFSA conclusion (EFSA, 2017) remain.

6. Recommendation

As regards the questions presented in the mandate on *P. chlororaphis* MA342, the PPR Panel recommends that:

- In order to reduce the uncertainties in relation to the potential exposure to metabolites, studies investigating the production of DDR and other relevant metabolites by *P. chlororaphis* MA342 (considering all life cycles) and their transformation under realistic conditions should be produced. Investigation of metabolism in plants and residue levels of viable and non-viable residues in treated crops following accepted test guidelines for the intended uses using validated analytical methods with sufficiently low LOQ will allow to reach definite conclusions in relation to the risk posed to consumers, workers and residents.
- In order to appropriately address the genotoxicity of DDR, genotoxic testing according to the Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Scientific Committee, 2011) and the Scientific Committee Guidance on aneugenicity assessment (ongoing, 2020) should be followed.

The PPR Panel notes that in order to finalise the risk assessment of other issues, not directly addressed by this statement, the following will need to be considered:

- As *P. chlororaphis* is not recommended for the QPS status and uncertainty remains on the taxonomic identity of the strain MA342, a risk assessment of the presence of *P. chlororaphis* strain MA342 in edible plant tissues would be necessary.
- Proper taxonomic characterisation of the strain following state of the art methodologies. Univocally determining the species to which strain MA342 pertains will allow to reduce the uncertainty associated with the use of data obtained on other strains of the same species.
- Antimicrobial production. The PPR panel notes that according to the applicant *P. chlororaphis* MA342 produces other secondary biologically active metabolites besides DDR. Safety in relation to these other secondary metabolites will need to be investigated and assessed.
- Antimicrobial resistance. The PPR Panel noted that according to the applicant *P. chlororaphis* MA342 has the ability to grow on chloramphenicol, ampicillin, spectinomycin and rifampicin, indicating the possibility that the strain contains antimicrobial resistance genes conferring resistance towards critically (ampicillin and rifampicin) and highly important (chloramphenicol) or important (spectinomycin) antimicrobials for human medicine (WHO, 2019). It should be further investigated that the strain MA342 does not contain antimicrobial resistance genes which could be transferred to other bacteria in the environment.

7. Documentation as provided to EFSA (if appropriate)

Dossier *P. chlororaphis* MA342. 10/2014. Submitted by Lantmännen BioAgri AB.

References

Note: A number of studies quoted here pertain to the dossier of *P. chlororaphis* MA342 and are unpublished. For these studies, an extensive summary can be found in Renewal Assessment Report (RAR) (The Netherlands, 2016). For transparency and easy comparison, these studies are quoted here in the same way they are quoted in the RMS RAR. The original complete studies have been examined by the PPR Panel for the elaboration of this statement.

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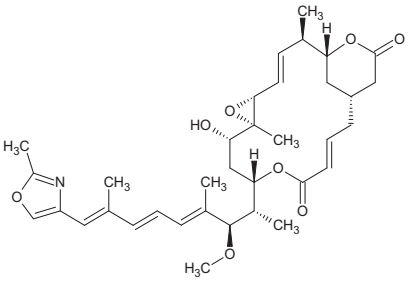
Abbreviations

bw	body weight
FISH	fluorescence *in situ* hybridisation
GLP	good laboratory practice
LOQ	limit of quantification
MRLs	maximum residue levels

NCIMB	National collection of industrial food and marine bacteria
NOEL	no observed effect level
PCE	polychromatic erythrocytes
PCR	polymerase chain reaction
PGPR	plant growth-promoting rhizobacteria
RAPD	randomly amplified polymorphic DNA
RAR	renewal assessment report
RFLP	Restriction Fragment Length Polymorphism
RMS	rapporteur Member State
QPS	Qualified presumption of safety
TTC	threshold of toxicological concern

Appendix A – Used compound codes

Code/trivial name	Chemical name/SMILES/InChI key	Structural formula
DDR **2,3-deepoxy-2,3-didehydro-rhizoxin**	(1*R*,2*R*,3*E*,5*R*,7*R*,8*S*,10*S*,13*E*,16*R*)-8-hydroxy-10-[(2*S*,3*R*,4*E*,6*E*,8*E*)-3-methoxy-4,8-dimethyl-9-(2-methyl-1,3-oxazol-4-yl)nona-4,6,8-trien-2-yl]-2,7-dimethyl-6,11,19-trioxatricyclo[14.3.1.$0^{5,7}$]icosa-3,13-diene-12,18-dione Cc1occ(n1)\C=C(/C)\C=C\C=C(/C)[C@pH](OC)[C@@H](C)[C@H]3OC(=O)C=CC[C@H]2CC(=O)O[C@H](C2)[C@H](C)C=C[C@H]4O[C@]4(C)[C@@H](O)C3 HZCHLCHTTHJHIL-NAGJTQNUSA-N	

Annex A – Original statement of the Standing working group on genotoxicity of the EFSA Scientific Committee on potential aneugenicity of DDR

Önfelt et al. (2000) Effects of the Rhizoxin derivative 2,3-deepoxy-2,3-didehydro-rhizoxin (DDR) on the mitotic spindle of V79 Chinese hamster cells.

V79 cells were exposed to DDR ranging from 2.5×10^{-12} to 2.5×10^{-5} M for 30 min. Three independent experiments were performed. Two hundred metaphases per concentration were scored for the analysis of mitotic abnormalities including lagging chromosomes, poorly organised metaphase plates, chromosomes scattered in the cytoplasm (overall defined as c-mitosis). Spindle structure was evaluated by immunofluorescent staining using anti-tubulin antibodies.

The authors reported limited solubility of the test item and referred that the applied concentrations were nominal. A statistically significant increase of c-mitosis was observed at 2.5×10^{-11} M ($n = 4$; $p = 0.0046$); at 5×10^{-11} M, no statistically significant increase was reported compared to control values (data not shown): the threshold for the effect is likely to be within or below this interval. Cytotoxicity was reported at $\geq 10^{-8}$ (data not shown).

The authors concluded DDR is an active microtubule inhibitor.

Comments to the study:

The experimental design seems appropriate to the aim of the study as well as the endpoint analysed.

The results are not shown in detail in tables or figures; few data have been provided to interpret the results (number of aberrant cells, the classification for c-mitosis).

The study provides limited evidence.

Abramsson-Zetterberg (2000). The genotoxic effect in mice of orally administered 2,3-deepoxy-2,3-didehydro-rhizoxin (DDR). Results from the *in vivo* micronucleus assay

An *in vivo* micronucleus test was performed in male NMRI mice treated by oral gavage with bacterial suspension of *Pseudomonas chlororaphis* containing DDR at 0.2, 2.05 and 18.6 mg/kg bw. Mice were sacrificed 38 and 62 h after treatment. Micronuclei were analysed in peripheral blood reticulocytes by flow cytometry. Colchicine was used as positive control (13 mg/kg bw). At least 70,000 cells were scored per animal. A statistically significant increase of MN was observed at the highest dose tested of DDR (18.6 mg/kg bw) at 38 h sampling time. (3.65 vs 1.7 MN/1,000 reticulocytes in treated and control groups, respectively; $p < 0.0067$). No increase of MN frequency was reported at the latest sampling time. Colchicine induced statistically significant increases in the frequency of micronuclei in reticulocytes at both sampling times. Bone marrow toxicity was not induced by DDR at any dose and any sampling times, as measured by the percentage of immature reticulocytes. Colchicine induced significant reduction of % polychromatic erythrocytes (PCE) at 38 and 62 h after treatment. A significant reduction of body weight was also observed in colchicine-treated mice, not detected after DDR treatment.

The authors concluded that very high concentrations of DDR (around 1,000 times higher than the DDR naturally produced in a culture of *Pseudomonas chlororaphis*) are required to induce detectable increase of chromosomal damage, evaluated as micronuclei frequency, in mice after oral administration. The assumption of an aneugenic mechanism, based on the results of Önfelt et al. (2000), was reinforced by the high mean DNA content of micronuclei induced by DDR (data not shown).

Comments to the study:

The experimental protocol applied essentially complies with the OECD guideline 474. The application of kinetochore staining or fluorescence *in situ* hybridisation (FISH) with pan-centromeric probe, providing the frequency of centromere-positive micronuclei, is required to confirm the aneugenic mechanism. In addition, a specific study design, based on a large number of doses is needed to establish the reference point for aneugenicity.