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24-Epibrassinolide

Volume 3 – B.9 (AS)

Rapporteur Member State: Austria

Version History

When	What
2018/05	Initial DAR

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B.9. ECOTOXICOLOGY DATA (CA 9)

This document presents ecotoxicological studies on the active substance 24-Epibrassinolide.

For the inclusion of the active substance 24-Epibrassinolide and the representative formulation Sunergist (0.01 % 24-Epibrassinolide) in Annex I, data to support the application for inclusion regarding ecotoxicology is provided in the following section. Studies, where available, are summarised under the respective data points. In some cases, public literature is used to address data points. In those cases where published literature is used to **address a data point**, an extended summary of the published literature is provided and cited (author, year), the full bibliographical information can be found under point B.9.10.

In the case where published literature is used to **scientifically justify** why a study was not deemed necessary to be conducted or as supporting information, only a superscript is referenced in the text, while full bibliographical information can be found in a respective footnote. Relevant literature from the EFSA- compliant literature search, which has to be evaluated on full-text level, is discussed under the respective data point under point B.9.10.

The applicant submitted an extensive introduction to brassinosteroids in support with the dossier of 24-Epibrassinolide. This general information was not evaluated in detail by RMS because it is not deemed necessary for the DAR preparation but is provided in Appendix I for completeness. Nonetheless a short version was extracted and accepted by RMS and is presented below to give an overview.

B.9.0. INTRODUCTION

Brassinosteroids, including 24-Epibrassinolide are naturally occurring, plant growth promoting molecules, present in higher plants, lower plants, including algae, mosses, the "living fossil" *Equisetum* as well as some fungi.[1,2,3] Brassinosteroids are present in all plant organs such as pollen, anthers, seeds, leaves, stems, roots, flowers, grains and fruits with the highest concentrations found in pollen, seeds and fruits and considered an obligatory plant constituent.[4,5]

Brassinosteroids are essential for normal plant growth and development. Those phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.[6] 24-Epibrassinolide elicits and activates the plant's self-defence mechanisms mediating the plant's resistance to unfavourable environmental conditions, (e.g.

[1] KCA 8/0001: Takatsuto, S., Abe, H., Gamoah, K. (1990): EVIDENCE FOR BRASSINOSTEROIDS IN STROBILUS OF EQUISETUM ARVENSE L. Report No.: na (092-059) Agricultural and Biological Chemistry, 1990, 54 (4), 1057-1059; Not GLP, published

[2] KCA 8/0011: Bajguz, A., Tretyn, A. (2003): THE CHEMICAL STRUCTURES AND OCCURRENCE OF BRASSINOSTEROIDS IN PLANTS. Report No.: na (092-145). Brassinosteroids. Chapter 1, 2003, 1-44. Not GLP, published.

[3] KCA 8/0012: Bajguz, A. (2011): BRASSINOSTEROIDS – OCCURRENCE AND CHEMICAL STRUCTURES IN PLANTS. In: Hayat, S., Ahmad, A.: BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE. Report No.: na (092-146). Springer Verlag, 2011, Chapter 1, 1-27, DOI 10.1007/978-94-007-0189-2_1; ISBN: 978-94-007-0188-5. Not GLP, published

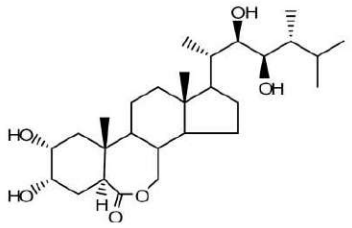
[4] KCA 8/0002: Zhu, J.-Y., Sae-Seaw, J., Wang, Z.-Y. (2013): BRASSINOSTEROID SIGNALLING. Report No.: na (092-165). Development, 2013, 140(8), 1615-1620; doi: 10.1242/dev.060590. Not GLP, published.

[5] KCA 8/0012: Codreanu, M.; Russinova, E. (2011): REGULATORY MECHANISMS OF BRASSINOSTEROID SIGNALING IN PLANTS. In: Hayat, S., Ahmad, A. (eds.): BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE. Report No.: na (092-146). Springer Verlag, 2011, Chapter 2, 29-56, DOI 10.1007/978-94-007-0189-2_2; ISBN: 978-94-007-0188-5. Not GLP, published

[6] KCA 8/0005: Kutschera, U., Wang, Z.-Y. (2012): BRASSINOSTEROID ACTION IN FLOWERING PLANTS: A DARWINIAN PERSPECTIVE. Report No.: na (092-036). Journal of Experimental Botany, 2012, 63 (10), 3511-3522; doi:10.1093/jxb/ers065. Not GLP, published

salinity, drought, cold and heat stress) and fungal diseases.[7] Application of brassinosteroids leads to a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, induction of protein synthesis and the production of various chemical defence compounds.[8]

Table 9.0-1 : Substances and metabolites of environmental relevance (structure, synonyms and codes)

Code	IUPAC name	Compound found in	Structural formula
24-Epibrassinolide	(22R,23R,24R)-2α,3α,22,23-tetrahydroxy-24-methyl-β-homo-7-oxa-5-cholestan-6-one	Environment (soil, surface water), plant, rat	
No relevant metabolites. Due to the natural occurrence of 24-Epibrassinolide and its metabolites, risk assessments for metabolites are not considered necessary as they are deemed to be covered by the parent.			

B.9.1. EFFECTS ON BIRDS AND OTHER TERRESTRIAL VERTEBRATES

B.9.1.1. Effects on birds

A waiver is requested by the applicant for the performance of standard terrestrial toxicity studies on birds because:

- No toxicity of 24-Epibrassinolide is expected because of the ubiquitous natural occurrence in plants.
- Therefore a constant natural exposure of birds to brassinosteroids through feed consumption is considered reasonable.
- Further, no toxicity was observed in studies with mammals (see KCA 8.1.2.1/0001).
- Brassinosteroids are essential for normal plant growth and development. Those phylogenetically ancient phytohormones evolved in the Pre-Cambrian, thus it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

The low toxicity to birds is, according to the applicant, further supported by Nasonov, I. *et al.*, 2016 (KCA 8.1.1/0001), who studied the immunostimulatory properties of Bravidefen, a brassinosteroid-based drug (24-Epibrassinolide in water-soluble form) in chickens.

Data point addressed:	CA B.9.1.1
Reference:	KCA 8.1.1/0001
Author(s) (year):	Nasonov, I.V., Likhacheva, M.I., Litvinovskaya, R., Sauchuk, A.L. (2016)
Title:	IMMUNOSTIMULATORY PROPERTIES OF BRAVIDEFEN, A BRASSINOSTEROID-BASED DRUG, IN CHICKENS
Published:	Yes
Published in:	23rd Conference on Isoprenoids and National Academy of Sciences of Belarus, Chemical Series 2016, N 3, 1-128 (Posters P99-100)
Test guideline used:	Not specified
Deviations:	Not applicable
GLP:	No

[7] KCA 8/0012: Kang, Y., Guo. S. (2011): ROLE OF BRASSINOSEROIDS ON HORTICULTURAL CROPS. In: Hayat, S., Ahmad, A. (eds.): BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE. Report No.: na (092-146). Springer Verlag, 2011, Chapter 9, 269-288, DOI 10.1007/978-94-007-0189-2_9; ISBN: 978-94-007-0188-5. Not GLP, published

[8] KCA 8/0091: Bajguz, A., Hayat, S. (2009): EFFECTS OF BRASSINOSTEROIDS ON THE PLANT RESPONSES TO ENVIRONMENTAL STRESSES. Report No.: na (092-133). Plant Physiology and Biochemistry, 2009, 47, 1-8; doi:10.1016/j.plaphy.2008.10.002. Not GLP, published

Acceptability:	**Yes - Additional information**
Summary provided by the applicant: 1 day old chickens were either watered at a dose of 1 ml per 100 g of live weight (concentration $0.25*10^{-4}$ M, group 1) or treated intranasally at a dose of 0.1 ml (group 2) after immunization by КМИЭВ-V104 Newcastle disease virus strain vaccine. After 7, 14, 21 and 28 days blood samples were analysed for the level of antibodies to Newcastle disease virus in comparison to a control group. Each group contained 10 chickens. Significant increase in antibody titer compared to control was found in group 1 after 14 days (2.6 $\log_2$ higher than control) and in group 2 after 21 days (1.59 $\log_2$ higher than control). Thus, Bravidefen showed immunostimulatory properties in chicken compared to vaccination only. Further, no effects on behaviour or toxicity were reported, which underlines that 24-Epibrassinolide is not harmful to birds.	
KCA 8.1.1/0001	**Comment RMS:** The referenced article does not specify whether it follows a certain test guideline. Only scarce details regarding study design are provided (exact uptake of the test substance via water, housing conditions etc.), no mortality is mentioned. The administered test substance is Bravidefen, a new drug prepared from a water-soluble form of brassinosteroid 24-Epibrassinolide for usage in the poultry industry as protective and stimulative medication. According to the study the birds were watered with 1 ml water/100g body weight with a Bravidefen concentration of $0.25*10^{-4}$ mol/L water, which equals 0.099163 mg Bravidefen/kg bw (based on the assumption that Bravidefen and 24-Epibrassinolide have the same molecular weight of 396.65 g/mol). This study is considered as additional information by RMS.

Overall RMS conclusion – Effects on birds:
The waiver for standard toxicity studies on birds is considered acceptable regarding the ubiquitous natural occurrence of brassinosteroids in plants, the resulting natural exposure to birds via food consumption and the low acute and long-term toxicity shown in mammals. Thus adverse effects posed by 24-Epibrassinolide are considered unlikely and the data requirement is considered sufficiently addressed.

B.9.1.1.1. Acute oral toxicity to Birds

Please refer to B.9.1.1.

B.9.1.1.2. Short-term dietary toxicity to birds

Please refer to B.9.1.1.

B.9.1.1.3. Sub-chronic toxicity and reproduction to birds

Please refer to B.9.1.1.

B.9.1.2. Effects on terrestrial vertebrates other than birds

B.9.1.2.1. Acute oral toxicity to mammals

According to the applicant no toxicity of 24-Epibrassinolide to mammals is expected because:

- 24-Epibrassinolide shows an ubiquitous natural occurrence in plants.
- Therefore a constant natural exposure of birds to brassinosteroids through feed consumption is considered reasonable.

- Brassinosteroids are essential for normal plant growth and development. Those phylogenetically ancient phytohormones evolved in the Pre-Cambrian, thus it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Nevertheless, an acute oral toxicity study with rats (OECD TG 423) has been conducted by [redacted] (2017) to assess the acute oral toxicity to mammals. It is provided in support of the assessment and has not been previously evaluated. This new study is submitted to comply with current data requirements and is summarised in Vol. 3 CA B6.

In addition, in the following publication by Khripach *et al.* (2000), the acute oral toxicity data of the Sanitary-Hygienic Institute of Belarus for 24-Epibrassinolide has been evaluated. The respective oral LD_{50}-value has been assessed in female mice to be greater than 1000 mg kg^{-1}, as well as greater than 2000 mg kg^{-1} in rats (male/female).

Data point addressed:	CA B.9.1.2.1
Reference:	KCA 8.1.2.1/0002
Author(s) (year):	Khripach, V., Zhabinskii, V., De Groot, A. (2000)
Title:	TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY
Published:	Yes
Published through:	Annals of Botany, 2000, 86, 441-447; doi:10.1006/anbo.2000.1227
Test guideline used:	Not specified
Deviations:	Not specified
GLP:	No
Acceptability:	**Yes - Additional information**

No summary was provided by the applicant, thus RMS extracted the relevant passage:

The metabolism of BS [brassinosteroids] *in mammals has not yet been investigated. It may be speculated, however, that a 'normal' catabolism of the steroidal skeleton will take place. Being normal constituents of practically all plants, BS have been, and are, consumed by mammals, and so additional harmful effects are not likely from their use in agriculture. This assumption is an important prerequisite for considering BS as ecologically safe, non-toxic chemicals for agriculture. However, confirmation of their safety can be obtained from toxicological studies.*
The acute toxicity data obtained at the Sanitary-Hygienic Institute of Belarus for 24-Epibrassinolide are: LD_{50} (orally) in mice (female) is more than 1000 mg kg^{-1};LD_{50} (orally and dermally) in rats (male/female) is more than 2000 mg kg^{-1}. Dermal toxicity in rats (male/female) is more than 2000 mg kg^{-1}. The formulation, Epin (0·025 % solution of 24-Epibrassinolide), in mice and rats (orally and dermally) has an LD_{50} of more than 5000 mg kg^{-1}. Repeated experiments confirmed the value of LD_{50} for 24-Epibrassinolide orally in mice and showed a value for Epin which was higher than 15 000 mg kg^{-1} (white rats, orally or intra-nasally). In concentrations of 0·2%, 24-Epibrassinolide did not irritate mucous membranes of rabbits' eyes; this compound, or a solution of Epin, did not irritate the skin. The Ames test for mutagenic activity carried out at the Scientific Research Center of Toxicologic and Hygienic Regulation of Biopreparations of Russia, with or without metabolic activation, was negative (Salmonella typhimurium TA 1534, TA 1537, TA 1950, TA 98, TA 100). In micro-nuclear or chromosome abberation tests (mice CBAB1/6) neither 24-Epibrassinolide nor Epin caused spontaneous mutations.
Complex biological testing on Tetrahymena pyriformis carried out at the Sanitary-Hygienic Institute of Belarus has confirmed the genetic safety of 24-Epibrassinolide and the absence of mutagenic activity over seven generations. In acute, subacute, and chronic experiments, 24-epibrassinolide showed low toxicity and very little cumulative effect. In prolonged experiments, 24-Epibrassinolide showed no toxicity but a pronounced adaptogenic effect (increasing adaptive ability of the population). Studies on fish toxicity showed no negative effects, but pronounced stimulative and toxico-protective properties (Vitvitskaya et al., 1997a,b).

KCA 8.1.2.1/0002	**Comment RMS:** The referenced paper does not specify whether it follows a certain test guideline. No details regarding study design or test guideline are provided. For 24-Epibrassinolide a LD_{50}-value (oral) for female mice is stated to be > 1000 mg/kg bw, for female & male rats (oral/dermal) a LD_{50}-value > 2000 mg/kg bw is referenced. This public literature paper is considered as additional information by RMS.

Overall RMS conclusion - Acute oral toxicity to mammals:
Based on the acute toxicity results indicated in the study by [redacted] (2017) a LD_{50} > 5000 mg/kg body weight can be set for 24-Epibrassinolide in mammals. This low toxicity is supported by the public literature data in Khripach *et al.* (2000).

Table 9.1.2-1 summarises the results of the available acute oral toxicity studies on mammals conducted with 24-Epibrassinolide.

Table 9.1.2-1: Acute oral toxicity of 24-Epibrassinolide on mammals

Test species	Study type	LD_{50} [mg a.s./kg bw]	Study
Rat	Acute oral toxicity study (OECD 423)	> 5000 [1)]	[redacted] (2017)

[1)] endpoint derived for females

B.9.1.2.2. Long-term and reproduction toxicity to mammals

A repeated dose 90-days oral toxicity study (OECD TG 408) and a prenatal developmental toxicity study (OECD TG 414) were performed with rats by [redacted] (2017) and [redacted] (2017) respectively, please refer to Vol. 3 CA B6 for further details.

Table 9.1.2-2: Information from the mammalian toxicology section, relevant to identify the ecotoxicologically relevant reproductive mammalian endpoint for 24-Epibrassinolide

Body weight change[1], behavioural effects and systemic toxicity[2]	28-day oral toxicity study (OECD 407): no study available. 90-day sub-chronic oral toxicity study (OECD 408): Rat male: NOAEL = 1000 mg/kg bw/d (based on body weigth) Rat female: NOAEL = 1000 mg/kg bw/d (based on body weigth) Multi-generation study (OECD 416): no study available. Developmental studies (OECD 414): Rat: maternal NOAEL = 1000 mg/kg bw/d (based on body weight and feed consumption)
Indices of gestation, litter size, pup and litter weight[3]	Multi-generation study (OECD 416): no study available. Developmental studies (OECD 414): Rat: developmental NOAEL = 1000 mg/kg bw/d
Indices of viability, pre- and post-implantation loss	Multi-generation study (OECD 416): no study available. Developmental studies (OECD 414): Rat: NOAEL = 1000 mg/kg bw/d
Embryo/foetal toxicity including teratological effects	Multi-generation study (OECD 416): no study available. Developmental studies (OECD 414): Rat: NOAEL = 1000 mg/kg bw/d
Number aborting and number delivering early	Multi-generation study (OECD 416): no study available. Developmental studies (OECD 414): Rat: NOAEL = 1000 mg/kg bw/d
Systemic toxicity and effects on adult body weight	Multi-generation study (OECD 416): no study available. Developmental studies (OECD 414): Rat: NOAEL = 1000 mg/kg bw/d

Indices of post-natal growth[4], indices of lactation and data on physical landmarks	Multi-generation study (OECD 416): no study available. Developmental studies (OECD 414): Rat: NOAEL = 1000 mg/kg bw/d
Survival and general toxicity up to sexual maturity	Multi-generation study (OECD 416): no study available. Developmental studies (OECD 414): Rat: NOAEL = 1000 mg/kg bw/d

[1] Included as an indicator for parental effects which may disrupt reproduction.
[2] Effects derived from absorption of the substance that causes modification of an organ or an apparatus (biochemical, physiological and/or morphological). Examples include behavioural or physiological impairment (e.g. reduced locomotive activity, altered reflexes).
[3] Any effects in foetal body weight should be evaluated in the context of all pertinent data including other developmental effects as well as maternal toxicity.
[4] For example body weight gain, ear and eye opening, tooth eruption, hair growth and effects on sexual maturation such as age and body-weight at vaginal opening or balano-preputial separation.

Overall RMS conclusion – Long-term toxicity to mammals:
Based on the prenatal developmental toxicity results indicated in the performed study (RCC Study number 6642) a NOAEL of 1000 mg/kg body weight can be set for 24-Epibrassinolide in mammals.
Table 9.1.2-6 summarises the results of the available developmental toxicity study on mammals conducted with 24-Epibrassinolide.

Table 9.1.2-3: Acute oral toxicity of 24-Epibrassinolide to mammals

Test species	Study type	Toxicity endpoint [mg a.s./kg bw]	Study
Rat	Repeated dose 90-days oral toxicity study (OECD 408)	NOAEL: 1000	[redacted] (2017)
Rat	Parental development toxicity study (OECD 414)	NOAEL: 1000	[redacted] (2017)

B.9.1.3. Active substance bioconcentration in prey of birds and mammals

According to the applicant no negative effects related to bioconcentration of 24-Epibrassinolide in birds and mammals is expected because:

- 24-Epibrassinolide exhibits an ubiquitous natural occurrence in plants.
- Therefore a constant natural exposure of birds to brassinosteroids through feed consumption is considered reasonable.
- Brassinosteroids are essential for normal plant growth and development. Those phylogenetically ancient phytohormones evolved in the Pre-Cambrian, thus it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Further, as the log Pow is below 3, no accumulation in the food chain will occur.

Overall RMS conclusion – Bioconcentration:
Since 24-Epibrassinolide has a log Pow of 2.0 (please refer to Vol. 3 CA Part B 2) which is < 3 and thus according to Commission Regulation (EU) 283/2013 an assessment of the potential risk posed by bioconcentration in the prey of birds and mammals is not considered necessary.

B.9.1.4. Other data on effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)

According to the applicant no negative effects of 24-Epibrassinolide in reptiles and amphibians are expected because:

- 24-Epibrassinolide exhibits an ubiquitous natural occurrence in plants.

- Therefore a constant natural exposure of birds to brassinosteroids through feed consumption is considered reasonable.
- Brassinosteroids are essential for normal plant growth and development. Those phylogenetically ancient phytohormones evolved in the Pre-Cambrian, thus it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Further, no negative effects on aquatic organisms, reptiles or amphibians were reported in open literature. Therefore adverse effects posed by 24-Epibrassinolide are considered unlikely.

Overall RMS conclusion – Other data on effects on terrestrial wildlife:
The data point is considered sufficiently addressed.

B.9.1.5. Potential for endocrine disruption

Brassinosteroids (BRs) are natural phytohormones, found in many plant species, with highest contents in seed, pollen and fruits.[9] As plant sterols they regulate hormonal balance, activation of protein and nucleic acid synthesis, enzyme activity, and growth promotion in plants. In addition, they mediate augmented resistance to unfavourable environmental factors, stress, and disease. Exogenous exposure has been connected to specific antioxidative effects, via improvement of synthesis of photosynthetic pigments and antioxidant enzymes activity.[10]
According to the adopted proposal for endocrine disrupting properties (SANTE/11992/2017 Rev.0), an endocrine disruptor shows an adverse effect in an intact organism or its progeny, has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system, and the adverse effect is a consequence of the endocrine mode of action.
There are no indications for an endocrine disruption potential of 24-Epibrassinolide from the available data. No adverse effects were observed in any of the available guideline studies. In addition, the literature search did not reveal any relevant publication demonstrating endocrine disrupting properties in mammalians.
Furthermore, 24-Epibrassinolide is not mentioned in any list of chemicals for which endocrine disrupting properties are proven or suspected (e.g. the lists of chemicals for Tier 1 screening in the EDSP of the US-EPA (2009)[11,12,13,14] or the list in the PIP report from 2009 (Position paper on the potential impact of proposed changes to EU pesticide regulations on ACP countries)[15].
A QSAR-Analysis by Wildemann (2015, reference KCA 5/114, please refer to Vol. 3 CA B6 B.6.8.1) showed that 24-Epibrassinolide and 28-Homobrassinolide have a positive mechanistic alert for endocrine disruption because they are strong binders and have an OH-group, but there is no alert for the endpoint profiler for the prediction of estrogen receptor binding affinity (rtER expert system ver. 1 - US EPA). Thus, the substances can be considered as non-endocrine disrupting.
In the literature search the public literature study by Rarova et al. (2012, reference KCA 5.8.2/0003, please refer to Vol. 3. CA B6 B.6.8.2) was found. Based on the results with human endothelial cells where in a receptor

[9] KCA 8/0012: Bajguz, A. (2011): BRASSINOSTEROIDS – OCCURRENCE AND CHEMICAL STRUCTURES IN PLANTS. In: Hayat, S., Ahmad, A.: BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE. Report No.: na (092-146). Springer Verlag, 2011, Chapter 1, 1-27, DOI 10.1007/978-94-007-0189-2_1; ISBN: 978-94-007-0188-5. Not GLP, published

[10] KCA 8.1.5/0002: Niu, J. (2016): EXOGENOUS APPLICATION OF BRASSINOLIDE CAN ALTER MORPHOLOGICAL AND PHYSIOLOGICAL TRAITS OF LEYMUS CHINENSIS (TRIN.) TZVELEV UNDER ROOM AND HIGH TEMPERATURES, Report No.: na (092-111), Chilean Journal of Agricultural Research, 2016, 76 (1), 27-33; doi: 10.4067/S0718-58392016000100004 Not GLP, published

[11] https://www.regulations.gov/document?D=EPA-HQ-OPPT-2004-0109-0080
[12] https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0477-0074
[13] https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-edsp-estrogen-receptor-bioactivity
[14] https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-tier-1-screening-determinations-and
[15] https://www.coleacp.org/en/system/files/file_fields/2016/05/11/eng-bd2520pip2520position2520paper2520potential2520impact2520proposed2520changes2520to2520eu2520pesticide-0.pdf

binding assay no activity was observed it is considered unlikely that 24-epibrassinolide will act as a modulator of steroid signalling *in vivo*.

There is at present no indication that 24-Epibrassinolide acts as an endocrine disruptor.

Overall RMS conclusion – Potential for endocrine disruption:
The argumentation above is accepted, potential endocrine disrupting effects posed by 24-Epibrassinolide are considered unlikely and the data requirement was sufficiently addressed.

B.9.2. EFFECT ON AQUATIC ORGANISMS

According to the applicant a low toxicity to aquatic organisms posed by the exposure to 24-Epibrassinolide is expected because:

- 24-Epibrassinolide shows an ubiquitous natural occurrence in plants.
- Brassinosteroids are essential for normal plant growth and development. Those phylogenetically ancient phytohormones evolved in the Pre-Cambrian, thus it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Nonetheless worst case surface water PECs were calculated in the fate section in Vol. 3 CP B.8. The maximum worst case PECsw (FOCUS Step 2) is 0.0232 µg a.s./L and the maximum PECsediment is 0.1632 µg a.s./kg.

B.9.2.1. Acute toxicity to fish

To determine the acute toxicity of 24-Epibrassinolide to fish, the following study was submitted and has not been previously evaluated.

Data point addressed:	CA B.9.2.1
Reference:	KCA 8.2.1/0001
Author(s) (year):	[redacted] (2017)
Title:	Acute Fish Toxicity Study in Freshwater Fish (Brachydanio rerio) with 24-Epibrassinolide (TGAI)
Laboratory report / project Number (Doc No.):	6122
Testing facility:	[redacted]
Published:	No
Test guideline used:	OECD No. 203
Deviations:	None
GLP:	Yes
Acceptability:	Yes

Executive Summary

Acute Fish Toxicity Study in Freshwater Fish (*Brachydanio rerio*) with 24-Epibrassinolide (TGAI) was performed as per OECD Guideline for the Testing of Chemicals, No. 203. Based on the test item solubility and range finding experiment results, Limit test was conducted with the concentration of 5 mg/L. Ten fresh water fish (*Brachydanio rerio)* were exposed for 96 hours to the limit test concentration (5 mg/L). Concurrent control group with ten fish was also maintained.
Prior to acclimation, length and body weight of ten fish were recorded. The fish were observed daily during the acclimatization period and all the fish were found to be normal.
The test item was formulated with exposure water. No mortality or sublethal effects were observed in control and fish exposed to 5 mg/L concentration throughout the experimental period. Thus the percent mortality at the end of 96 hour was recorded to be 0% in control and 5 mg/L concentration.
No toxic signs were observed in control and fish exposed to 5 mg/L concentration. Therefore, the endpoint was determined to be:
LC_{50} > 5 mg/L

Analytical verification of the test item at a concentration of 5 mg/L in the exposure water was performed. The mean recovery data is given below:

- '0' hour – mean recovery results (5 mg/L): 98.27 %
- '96' hour – mean recovery results (5 mg/L): 97.84%

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material:

Test Material:	24-Epibrassinolide (TGAI)
Description:	Odorless Oyster White Solid Powder
Lot/Batch #:	002-20150112
Purity:	91.2% w/w

2. Vehicle and control:

Control:	Exposure water

3. Test animals:

Species:	Zebrafish (*Brachydanio rerio*)
Age:	not reported
Mean length:	2.3 – 2.9 cm
Mean weight:	0.24 – 0.29 g
Source:	[redacted]
Acclimatisation period:	8 days under laboratory conditions. Only fish without any visible signs of illness were used for the study
Diet:	Feed was withdrawn approximately 24 hours before test. fish were not fed during the test
Water:	Aquaguard water and Reverse osmosis water was blended in the ratio of 1:1 and aerated before use. pH was 7.2 (±0.5) for control and treatment group in range finding and limit test
Test unit :	Glass aquarium
Volume of test solution :	10 L
Environmental conditions/ water quality range finding test:	
Temperature:	22.2 – 22.4 °C
Dissolved oxygen:	70.2% - 90.4%
pH:	6.94 - 7.64
Hardness:	220 mg/L as CaCO3
Photoperiod:	Light cycle of 12 hours light and 12 hours dark
Environmental conditions/ water quality limit test::	
Temperature:	22.3 – 22.4 °C
Dissolved oxygen:	71.8% - 89.5%
pH:	6.98 – 7.64
Hardness:	220 mg/L as CaCO3
Photoperiod:	Light cycle of 12 hours light and 12 hours dark

4. Test conditions:

Static for 96 h

B. STUDY DESIGN AND METHODS

1. In life dates: 24.05.2016 – 13.07.2016

2. Preparation of test solutions

Solubility analysis

100 mg of test item was received and from that approximately 10 mg of test item was reweighed and to that 1 L of exposure water was added. The test item particles were settled at the bottom and it was not found to be soluble even after ultrasonicating for 30 minutes. Therefore, from the remaining test item approximately 5 mg was reweighed and to that 1L of exposure water was added, ultrasonicated for 15 minutes and the test item appeared to be soluble. As 5 mg/L concentration

was soluble, an attempt was taken to check the solubility limit further for which the test item was gradually increased up to 8 mg/L concentration. However, test item particles appeared and were found to be not soluble, confirmed by the analytical results.

Range finding test
The dose formulations were prepared shortly before exposure. Required test concentrations were 1, 2, 3, 4 and 5 mg/L and all the test concentrations were prepared for 10 L. 1000 mL of exposure water was taken from the respective labeled tanks and 10.02, 20.05, 30.06, 40.01 and 50.02 mg of test item was added and ultrasonicated for 15 minutes and transferred back to the respective labeled tanks. A glass rod was used to mix the solution thoroughly in each tank.

Limit test
The dose formulation was prepared shortly before exposure. Required test concentration was 5 mg/L and the test concentration was prepared for 10 L. 1000 mL of exposure water was taken from the respective tank to which 50.02 mg of the test item was added, ultrasonicated for 15 minutes and then the test solution was transferred back into the respective labelled tank. Glass rod was used to mix the test solution well.

3. Test design and test procedure

The acute toxicity of 24-Epibrassinolide (TGAI) to zebrafish (*Brachydanio rerio*) was determined in a static, 96-hour test. Treatments consisted of an exposure water control and 10 fish per concentration (1, 2, 3, 4 and 5 mg test item/L). 10 fish were exposed to each concentration and control in the range finding test. In the limit test, 10 fish were exposed to 5 mg/L test item and control.

Fish were observed at 3, 6, 24, 48, 72 and 96 hours At 96 hour of exposure, cumulative mortality at test concentration was determined. At the end of the experiment, surviving fish were sacrificed using MS222 (1g/L distilled water) and disposed as toxic waste disposal.

4. Analytical Methods

During the conduct of the limit test, 100 ml of sample was collected on day the '0' day of exposure and on completion at '96' hour from control and the limit test concentration (5 mg /L) and the same were sent to [redacted] for concentration verification. The samples were analysed by a validated HPLC-method with PDA detector.

5. Statistics

Statistical analysis was not used as the study is concluded with Limit test. No mortality or other signs of toxicity were observed.

II. RESULTS AND DISCUSSION

A. Analytical Results

Measured concentrations of 24-Epibrassinolide (TGAI) ranged from 97 to 98 % of nominal concentrations. Under the test conditions the test item was sufficiently stable during the test period of 96 hours (see Table 9.2.1-1).

Table 9.2.1-1: Analytical determination of concentrations tested

Nominal Concentration of test item [mg/L]	**Mean Measured Concentrations of test item [mg/L]**	**Test Concentration [% of nominal] samples taken after**	
		0 h	96 h
Control	Control	-	-
5	4.913	98.27	97.84

Due to these analytical results, the nominal concentration was used to describe toxicity.

B. Water quality

Water quality parameters like temperature, dissolved oxygen and pH were within acceptable limits.

C. Biological test results

Summaries of cumulative mortality for range finding and limit test as well as sublethal effects for both tests are presented in Table 9.2.1-2, Table 9.2.1-3; Table 9.2.1-4 and Table 9.2.1-5 respectively.

There was no mortality observed in the range finding an limit test. Further, no sublethal effects were observed in neither of the tests.

Table 9.2.1-2: Observed mortality of zebrafish, *Brachydanio rerio,* exposed to 24-Epibrassinolide (TGAI) for 96 hours in a static range finding test

Mean Measured Concentrations 24-Epibrassinolide (TGAI) [mg/L]	**Cumulative Mortality (No. affected / No. at test start)** **Time**				
	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Control	0/10	0/10	0/10	0/10	0/10
1	0/10	0/10	0/10	0/10	0/10
2	0/10	0/10	0/10	0/10	0/10
3	0/10	0/10	0/10	0/10	0/10
4	0/10	0/10	0/10	0/10	0/10
5	0/10	0/10	0/10	0/10	0/10

Table 9.2.1-3: Observed mortality of zebrafish, *Brachydanio rerio,* exposed to 24-Epibrassinolide (TGAI) for 96 hours in a static limit test

Mean Measured Concentrations 24-Epibrassinolide (TGAI) [mg/L]	**Cumulative Mortality (No. affected / No. at test start)** **Time**				
	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Control	0/10	0/10	0/10	0/10	0/10
5	0/10	0/10	0/10	0/10	0/10

Table 9.2.1-4: Observed sublethal effect of zebrafish, *Brachydanio rerio,* exposed to 24-Epibrassinolide (TGAI) for 96 hours in a static range finding test

Mean Measured Concentrations 24-Epibrassinolide (TGAI) [mg/L]	**Sublethal Effects (No. affected / No. at test start)** **Time**				
	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Control	0/10	0/10	0/10	0/10	0/10
1	0/10	0/10	0/10	0/10	0/10
2	0/10	0/10	0/10	0/10	0/10
3	0/10	0/10	0/10	0/10	0/10
4	0/10	0/10	0/10	0/10	0/10
5	0/10	0/10	0/10	0/10	0/10

Table 9.2.1-5: Observed sublethal effect of zebrafish, *Brachydanio rerio,* exposed to 24-Epibrassinolide (TGAI) for 96 hours in a static limit test

Mean Measured Concentrations 24-Epibrassinolide (TGAI) [mg/L]	**Sublethal Effects (No. affected / No. at test start)** **Time**				
	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Control	0/10	0/10	0/10	0/10	0/10
5	0/10	0/10	0/10	0/10	0/10

D. Criteria for validity of the test

The study is considered valid since the following conditions were fulfilled:

- No mortality observed in control
- Constant conditions were maintained throughout the test

- The dissolved oxygen concentration was above 60 % of the air saturation value throughout the test and ranged from 76.8 to 92.5 % in control and 70.2 to 90.4 % in the treatment
- The concentration of test item tested was satisfactorily maintained and above 80 % of the nominal concentration throughout the test.

III. CONCLUSIONS

The freshwater fish, *Bracydanio rerio*, was exposed to 24-Epibrassinolide (TGAI) under static conditions for 96 hours at the test item's solubility limit of 5 mg/L. Based on the test results, LC_{50} of 24-Epibrassinolide(TGAI) observed over a period of 96 hour for freshwater fish (*Brachydanio rerio*) was recorded to be greater than 5 mg/L. The NOEC and LOEC were therefore determined to be 5 mg/L and > 5 mg/L.

KCA 8.2.1/0001	**Comment RMS:** The study is relevant and reliable. The validity criteria according OECD 203 (1992) are met. No mortality occurred in the acute limit test, therefore the endpoint is confirmed: **LD_{50} > 5 mg a.s./L (nom)**

As also reported in the solubility test of the above mentioned study, 24-Epibrassinolide is only water-soluble in very small amounts. The reported water solubility is 3.8 mg/L (at 20°C in purified water), please refer to Vol. 3 CA Part B 2. Therefore, high concentrations of dissolved 24-Epibrassinolide in water are very unlikely. Further, and even more importantly, 24-Epibrassinolide is used in fish farms for industrial production in Russia as described in a review by Zhabinskii *et al.*, 2015.

Data point addressed:	CA B.9.2.1
Reference:	KCA 8.2.1/0002
Author(s) (year):	Zhabinskii, V.N., Khripach, N.B., Khripach, V.A (2015)
Title:	STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM
Published:	Yes
Published in:	Steroids, 2015, 97, 87-97; doi: 10.1016/j.steroids.2014.08.025
Test guideline used:	Not applicable
Deviations:	Not applicable
GLP:	Not GLP
Acceptability:	**Yes - Additional information**

Summary by the applicant:

"As described in a review by Zhabinskii et al, 2015, 24-Epibrassinolide treatment of fingerlings of diverse fish species (e.g. black sea salmon, carp, Russian sturgeon and silver carp) lead to significantly less negative effects through toxicants ($CuSO_4$, phenol) contained in the water and treatment of sturgeon eggs with 24-Epibrassinolide was found to increase fecundation, hatching and larvae/fingerling survival (higher resistance to stress). This effect was also found for phytophaguous fishes (grass carp and silver carp)."

Relevant part extracted by RMS and shown for convenience:

„Intensive studies of BS effects on fishes started in the second half of the 1990s [49] in Russia and within a short period of time have led to a practical application of the research outcomes [50¬56] in fish farming for the protection of embryos, larvae and finger-lings from unfavorable environmental ecological conditions and for increasing fish production [57]. The first experiments were carried out with Russian sturgeon Atipenser gueldenstaedti belonging to a unique group of bony fish. Sturgeon fingerlings treated with epi-brassinolide (EB1) solution ($10'^{4}$mg/L) prior to their exposure to toxicants (such as $CuS0_4$, phenol, or the detergent) were significantly less negatively influenced by the toxicants than untreated control 149]. This could be seen from the higher abilities of finger-lings with regard to movements, reactions to a sonic signal, resistance to a current and training. Similar results were obtained for Black Sea salmon, carp, crucian and silver carp [53]. Analysis of physiology-biochemical parameters of treated and control fishes showed that EB1 possessed antioxidant properties [49] and stabilized hematoencephalic and histohematogenous barriers [58,59]. Prolonged exposure of silver carp to copper or organic toxicants resulted in an increase in erythrocyte catalase activity. Prior application of EB1 returned it to nearly control values [60]. Immersing Siberian sturgeon in an EB1 solution led to an increase of cerulo-plasmin level (over 500% higher on the fifth week of the study) [51]. This is an indication of EB1 immunostimulatory properties which might have resulted through the effect of activated leukocytes on hepatocytes. A significant decrease of hemoglobin content in the

blood was observed under the action of the toxicants. This parameter was greatly improved in EBl-treated fishes [49]. *Lipid peroxidation is the process of oxidative degradation of lipids that becomes more intense under stress conditions. BS were shown to decrease in plants the accumulation of malonic dialde-hyde [61], which is the most important product of lipid degradation. The same tendency was observed in fishes exposed to copper, phenol or detergent toxicants [53]. Level of malonic dialde-hyde in fishes treated by EB1 and toxicants showed no statistical difference with the control (toxicant-untreated) group. At the same time, in the toxicant-treated group without EBl level of malonic dialdehyde was significantly higher.* *A pronounced effect of BS on fish reproduction was found [55,62-64]. Thus, treatment of Russian sturgeon eggs with EBl gave a significant increase of a fecundation, hatching and larvae survival [62], The EBl-treated eggs produced the fingerlings with better morphological characteristics and resistance to stress. Immersing sturgeon fish larvae in EBl solution led to better survival of the fingerlings and to increase their body weight. The same effects were also seen on phytophagous fishes (grass carp and silver carp) [63,64], Treatment with EBl of Spermatozoons of Russian sturgeon enhanced their activity and viability, especially in the case of Spermatozoons reactivated after cryoconservation [55].“*	
KCA 8.2.1/0002	**Comment RMS:** The referenced paper does not specify whether it follows a certain test guideline. No details regarding study design or test guideline are provided. Nonetheless it is seen to support the assumption of low toxicity posed by brassinosteroids to fish. This public literature paper is considered as additional information by RMS.

Overall RMS conclusion – Acute toxicity to fish:

Table 9.2.1-6 summarises the results of the available acute toxicity study conducted with 24-Epibrassinolde ([redacted] 2017). The relevant endpoint to be used for the acute risk assessment of 24-Epibrassinolide is the 96-hours LC_{50} > 5 mg a.s./L. This low toxicity is supported by the public literature data in Zhabinskii *et al.* (2015).

Table 9.2.1-6: Acute toxicity of 24-Epibrassinolide to fish

Group	Test substance	Time scale	Endpoint	Endpoint [mg a.s./L]	Reference
Zebrafish (*Danio rerio*)	24-Epibrassinolide	Acute, 96 h, static	Mortality, LC_{50}	> 5.0 (nom)	[redacted] (2017)

B.9.2.2. Long-term and chronic toxicity to fish

A waiver is requested by the applicant for the performance of long-term and chronic toxicity studies on fish because:

- Due to the ubiquitous occurrence of 24-Epibrassinolide and other brassinosteroids in plants, the natural exposure of aquatic organisms to the substance, as well as the low acute toxicity for fish, testing of fish early-life stage toxicity is not considered necessary.
- Further, due to the low solubility of 24-Epibrassinolide in water and the readily uptake of free 24-Epibrassinolide by plants as well as the low application rate, no notable concentrations in surface water after application are expected and therefore further testing was not conducted.
- In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Overall RMS conclusion – Long-term toxicity to fish:

No studies were submitted by the notifier to address the long-term effects on fish. No explicit data was presented by the applicant regarding natural background concentrations of 24-Epibrassinolide in water. In a public literature study by Hassett et al. (1977, reference KCA 8.2.5.4/0001, evaluated under point CA B.9.2.5.3) sterols were found in two North American lakes, with lake water sterol concentrations ranging from 0.7 – 3 µg/L. Although brassinosteroids are not explicitly mentioned, the referenced sterols are considered to have a close structural relation or represent even precursors of brassinosteroids and are therefore seen suitable to serve as a proxy to estimate the order of magnitude of 24-Epibrassinolide concentration in water. A worst case surface

water PEC of 0.0232 µg a.s./L (FOCUS Step 2) was calculated in the fate section in Vol. 3 CP B.8. This supports the assumption that the natural exposure to 24-Epibrassinolide can be considered to be higher than the exposure following an application of the active substance in the form of a plant protection product. Thus the argumentation to waive the chronic fish studies is considered acceptable due to the expected natural occurrence of brassinosteroids and other sterols in the environment of aquatic organisms. Further a low acute toxicity was demonstrated in fish and a generally low water solubility of 3.8 mg/L (please refer to Vol. 3 CA Part B 2) is reported for the active substance. In conclusion, adverse long-term effects to fish posed by 24-Epibrassinolide are considered unlikely and the data requirement was sufficiently addressed.

B.9.2.2.1. Fish early life stage test

Please refer to CA B9, B.9.2.2.

B.9.2.2.2. Fish full-life-cycle test

A fish full life cycle study is not required, since 24-Epibrassinolide is neither considered to have a potential for bioaccumulation (see CA, B.9.2.2.3) nor it is a potential endocrine disruptor (see CA, B.9.2.3).

B.9.2.2.3. Bioconcentration in fish

Due to the moderate lipophilic properties of 24-Epibrassinolide with a log Pow = 2.0 (please refer to Vol. 3 CA B2), high bioaccumulation and bioconcentrations in fish are not to be expected and testing is not considered necessary according to Regulation (EC) 1272/2008 (trigger log Pow ≥ 4) and Commission Regulation (EU) 283/2013 (trigger log Pow ≥ 3). 24-Epibrassinolide occurs ubiquitously in plants and is used in fish farms in Russia to improve water quality (Zhabinskii *et al.* (2015), reference KCA 8.2.1/0002, evaluated under CA B.9.2.1). Brassinosteroids have been found in aquatic organisms such as algae (e.g. *Chlorella vulgaris*) and fish are naturally exposed to these organisms and substances.

B.9.2.3. Potential for endocrine disruption

Brassinosteroids (BRs) are natural phytohormones, found in many plant species, with highest contents in seed, pollen and fruits.[16] As plant sterols they regulate hormonal balance, activation of protein and nucleic acid synthesis, enzyme activity, and growth promotion in plants. In addition, they mediate augmented resistance to unfavourable environmental factors, stress, and disease. Exogenous exposure has been connected to specific antioxidative effects, via improvement of synthesis of photosynthetic pigments and antioxidant enzymes activity.[17]

According to the adopted proposal for endocrine disrupting properties (SANTE/11992/2017 Rev.0), an endocrine disruptor shows an adverse effect in an intact organism or its progeny, has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system, and the adverse effect is a consequence of the endocrine mode of action.

There are no indications for an endocrine disruption potential of 24-Epibrassinolide from the available data. No adverse effects were observed in any of the available guideline studies. In addition, the literature search did not reveal any relevant publication demonstrating endocrine disrupting properties in mammalians.

Furthermore, 24-Epibrassinolide is not mentioned in any list of chemicals for which endocrine disrupting properties are proven or suspected (e.g. the lists of chemicals for Tier 1 screening in the EDSP of the US-EPA

[16] KCA 8/0012: Bajguz, A. (2011): BRASSINOSTEROIDS – OCCURRENCE AND CHEMICAL STRUCTURES IN PLANTS. In: Hayat, S., Ahmad, A.: BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE. Report No.: na (092-146). Springer Verlag, 2011, Chapter 1, 1-27, DOI 10.1007/978-94-007-0189-2_1; ISBN: 978-94-007-0188-5. Not GLP, published

[17] KCA 8.1.5/0002: Niu, J. (2016): EXOGENOUS APPLICATION OF BRASSINOLIDE CAN ALTER MORPHOLOGICAL AND PHYSIOLOGICAL TRAITS OF LEYMUS CHINENSIS (TRIN.) TZVELEV UNDER ROOM AND HIGH TEMPERATURES, Report No.: na (092-111), Chilean Journal of Agricultural Research, 2016, 76 (1), 27-33; doi: 10.4067/S0718-58392016000100004 Not GLP, published

(2009)[18,19,20,21] or the list in the PIP report from 2009 (Position paper on the potential impact of proposed changes to EU pesticide regulations on ACP countries)[22].

A QSAR-Analysis by Wildemann (2015, reference KCA 5/114, please refer to Vol. 3 CA B6 B.6.8.1) showed that 24-Epibrassinolide and 28-Homobrassinolide have a positive mechanistic alert for endocrine disruption because they are strong binders and have an OH-group, but there is no alert for the endpoint profiler for the prediction of estrogen receptor binding affinity (rtER expert system ver. 1 - US EPA). Thus, the substances can be considered as non-endocrine disrupting.
In the literature search the public literature study by Rarova et al. (2012, reference KCA 5.8.2/0003, please refer to Vol. 3. CA B6 B.6.8.2) was found. Based on the results with human endothelial cells where in a receptor binding assay no activity was observed it is considered unlikely that 24-epibrassinolide will act as a modulator of steroid signalling *in vivo*.

There is at present no indication that 24-Epibrassinolide act as an endocrine disruptor.

Overall RMS conclusion – Potential for endocrine disruption:
The argumentation above is accepted, potential endocrine disrupting effects posed by 24-Epibrassinolide are considered unlikely and the data requirement was sufficiently addressed.

B.9.2.4. Acute toxicity to aquatic invertebrates

The applicant provided the following general statement regarding the acute toxicity to aquatic invertebrates:

"In general, Harvey et al. (1987)[23] found that ring saturated stanols, similar to 24-Epibrassinolide, appear to pass through the gut of Calanus helgolandicus quantitatively after feeding on the dinoflagellate Scrippsiella trochoidea, whereas unsaturated sterols were found to be removed from the dinoflagellate at all dietary levels. Stanol content in faecal pellets of the copepod was increased compared to the initial content in the food source Scrippsiella trochoidea, indicating that unsaturated sterols were metabolised to saturated stanols. It is therefore likely that aquatic invertebrates are not influenced by substances like 24-Epibrassinolide, as they are not unsaturated and therefore not easily metabolised. For Daphnids, Martin-Creuzburg & Von Elert (2004)[24] reported no effect on growth and development in studies supplementing completely saturated sterols to the diet of daphnids. These findings also correspond to the study assessing the toxicity of 24-Epibrassinolide to Daphnia magna (see study below.)
In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones."

B.9.2.4.1. Acute toxicity to *Daphnia magna*

The following study was conducted to assess the acute toxicity of 24-Epibrassinolide to *Daphnia magna*. In the following, the results will be presented.

[18] https://www.regulations.gov/document?D=EPA-HQ-OPPT-2004-0109-0080
[19] https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0477-0074
[20] https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-edsp-estrogen-receptor-bioactivity
[21] https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-tier-1-screening-determinations-and
[22] https://www.coleacp.org/en/system/files/file_fields/2016/05/11/eng-bd2520pip2520position2520paper2520potential2520impact2520proposed2520changes2520to2520eu2520pesticide-0.pdf
[23] KCA 8.2.4/0001: Harvey, H.R., Eglinton, G., O'Hara, S.C.M., Dorner, E.D.S. (1987): BIOTRANSFORMATION AND ASSIMILATION OF DIETARY LIPIDS BY CALANUS FEEDING ON A DINOFLAGELLATE, Report No.: na (092-166), Geochimica et Cosmochimica Acta, 1987, 51, 3031-3040, Not GLP, published

[24] KCA 8.2.4/0002: Martin-Creuzburg, D., Von Elert, E. (2004): IMPACT OF 10 DIETARY STEROLS ON GROWTH AND REPRODUCTION OF DAPHNIA GALEATA, Report No.: na (092-167), Journal of Chemical Ecology, 2004, 30(3), 483-500, Not GLP, published

GLP studies:

Data point addressed:	CA B.9.2.4.1
Reference:	KCA 8.2.4.1/0001
Author(s) (year):	Matlock, D., Moore, S. (2017)
Title:	Acute Toxicity of 24-Epibrassinolide to *Daphnia magna* Under Static Conditions
Laboratory report / project Number (Doc No.):	115SRUS16C0107
Testing facility:	SynTech Research Laboratory Services, LLC
Published:	No
Test guideline used:	OCSPP Guideline 850.1010 (1996 *draft*), OECD Guideline 202 (2004)
Deviations:	None
GLP:	Yes
Acceptability:	**Yes**

Executive Summary

The acute toxicity of 24-Epibrassinolide to *Daphnia magna*, was determined in a static, 48-hour test. Treatments consisted of a dilution water control and the nominal concentrations of 0.250, 0.500, 1.00, 2.00, 4.00 mg a.i./L.
Geometric mean concentrations were control, 0.218, 0.501, 0.982, 1.85, and 2.86 mg a.s./L. Day 0 recoveries ranged from 95% to 114% of nominal values. Geometric mean values from days 0 and 2 ranged from 72% to 100% of nominal values. Results are based on geometric mean measured test concentrations.
The 48-hour EC_{50} was > 2.86 mg a.s./L, based on geometric mean measured test concentrations, corresponding to > 4.00 mg a.s./L nominal concentrations, the solubility limit under test conditions. Based on immobilization, the 48-hour NOEC is 0.982 mg a.s./L and the 48-hour LOEC was 1.85 mg a.s./L

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material:

Test Material:	24-Epibrassinolide
Description:	Oyster white powder
Lot/Batch #:	002-20150112
Purity:	91.2 % w/w

2. Vehicle and control:

Control:	dilution medium (water)

3. Test animals:

Species:	Water Flea (*Daphnia magna*)
Age:	< 24 hours
Source:	Environmental Consulting & Testing (ECT), Superior, Wisconsin 54880, USA.
Diet:	Water flea were not fed throughout the exposure period.
Test unit:	250 mL borosilicate glass beakers
Number of replicates:	4 per level
Organisms per replicate:	5
Environmental conditions/ water quality:	
Temperature:	20.4-20.8 °C
Dissolved oxygen:	8.6 mg/L
pH:	8.1-8.4
Hardness:	160-200 mg/L as CaCO3
Photoperiod:	16 hours light / 8 hours dark with 30 minute dawn/dusk transition period; Cool white fluorescent (429-536 lux)

4. Test conditions: static

B. STUDY DESIGN AND METHODS

1. In life dates: January 2017

2. Preparation of test solutions

The test solutions were prepared by adding an appropriate amount of the test to a volumetric flask and bringing to volume with dilution water. Test solutions were sonicated for 4 hours and inverted several times to assure proper mixing. The following test concentrations were made via serial dilution starting with the 4.00 mg a.i./L test concentration: 0.250, 0.500, 1.00, 2.00 mg a.i/L. All vessels were brought to volume with hard processed water after adding the appropriate volume of the next higher test solution and inverted several times.

3. Test design and test procedure

The acute toxicity of 24-Epibrassinolide to *Daphnia magna*, was determined in a static 48-hour test. Treatments consisted of a dilution water control and the nominal concentrations of 0.250, 0.500, 1.00, 2.00, 4.00 mg a.i./L. and were tested with 4 replicates each and each containing 5 water fleas. All concentrations were observed at 4, 24 and 48 hours for immobility and other abnormal effects.

4. Statistics

Since none of the observed immobility data were 50% or above, no statistical analysis was performed to calculate the EC_{50}. The NOEC and LOEC values based on immobility were calculated using CETIS statistical software and were determined by the characteristics of the data, i.e. the number of concentrations in which immobilizations were between 0 and 100 percent and the 95% confidence intervals.

II. RESULTS AND DISCUSSION

A. Analytical Results

The measured concentrations of 24-Epibrassinolide was determined in water samples taken from batch solutions at each concentration on Day 0 and from composites of replicates at each concentration on Day 2. Analytical results are reported in Table 9.2.4-1.

Table 9.2.4-1: Analytical results for 24-Epibrassinolide in a 48 h *Daphnia magna* study

Nominal test concentration [mg a.i/L]	Initial measured test concentrations [mg a.i/L]	48h measured test concentration [mg a.i/L]	Geometric mean measured test concentration [mg a.i/L]
Control	Control (< LOQ)	Control (< LOQ)	Control (< LOQ)
0.25	0.237	0.200	0.218
0.5	0.572	0.439	0.501
1.0	1.11	0.869	0.982
2.0	2.24	1.52	1.85
4.0	3.84	2.14	2.86

B. Water quality and validity

Chemical and physical parameters (dissolved oxygen concentration, pH, temperature) in the definitive test were within expected ranges.

Validity criteria for this study were met, as 0% of the daphnids showed immobilization or other signs of disease or stress (validity criteria: ≤10%) and dissolved oxygen concentrations at the end of the test was 8.6 mg/L (validity criteria: ≥ 3 mg/L) in control vessels.

C. Biological test results

Observations after 4, 24 and 48 hours showed that daphnids in the controls and in the three lowest test levels were normal. Sublethal effects occurred in the two highest test levels. Detailed results are reported in Table 9.2.4-2.

Table 9.2.4-2: Summary of effects in *Daphnia magna* exposed to 24-Epibrassinolide

Geometric Mean	Hour 4	24 Hour	48 Hour

Measured Concentration (mg a.s./L)	Immob.	Obs	Immob.	Obs	Immob.	Obs
Control	0 (0%)	20 N	0 (0%)	20 N	0 (0%)	20 N
0.218	0 (0%)	20 N	0 (0%)	20 N	0 (0%)	20 N
0.501	0 (0%)	20 N	0 (0%)	20 N	0 (0%)	20 N
0.982	0 (0%)	20 N	0 (0%)	20 N	0 (0%)	20 N
1.85	0 (0%)	20 N	0 (0%)	20 N	2 (10%)	2 I; 4 Q; 14 N
2.86	0 (0%)	20 N	0 (0%)	20 N	4 (20%)	4 I; 13 Q; 3 N

Immob. = Cumulative number of organisms not able to swim after 15 seconds of gentle agitation of the test solution.
Obs = Observations (number of individuals observed alive plus observation)
I = Immobilized
N = Normal
Q = Quiescent

III. CONCLUSIONS

The 48-hour EC_{50} was > 2.86 mg a.s./L, based on geometric mean measured test concentrations, corresponding to > 4.00 mg a.s./L nominal concentrations, the solubility limit under test conditions. Based on immobilization, the 48-hour NOEC was 0.982 mg a.s./L and the 48-hour LOEC was 1.85 mg a.s./L.

KCA 8.2.4.1/0001	**Comment RMS:** The study is relevant and reliable. The validity criteria according OECD 202 (2004) are met. The study endpoint is confirmed: **EC_{50} > 2.86 mg a.s./L (mm)**

Overall RMS conclusion – Acute toxicity to aquatic invertebrates

Table 9.2.4-3 summarises the result of the available acute invertebrate toxicity study conducted with 24-Epibrassinolide (Matlock & Moore, 2017). The relevant endpoint to be used for the acute risk assessment of 24-Epibrassinolide is the 48-hours EC_{50} > 2.86 mg a.s./L, based on geometric mean measured test concentrations.

Table 9.2.4-3: Acute toxicity of 24-Epibrassinolide to aquatic invertebrates

Group	Test substance	Time scale	Endpoint	Endpoint [mg a.s./L]	Reference
Daphnia magna	24-Epibrassinolide	Acute, 48 hr, static	Mortality, EC_{50}	> 2.86 (mm)	Matlock, D. & Moore, S. (2017)

B.9.2.4.2. Acute toxicity to an additional aquatic invertebrate species

Not required.

B.9.2.5. Long-term and chronic toxicity to aquatic invertebrates

A waiver is requested by the applicant for the performance of long-term and chronic toxicity studies on aquatic invertebrates because:

- Due to the ubiquitous occurrence of 24-Epibrassinolide and other brassinosteroids in plants, the natural exposure of aquatic invertebrates to brassinosteroids and brassinosteroid-synthesising aquatic organisms such as *Chorella vulgaris*, as well as the low acute toxicity for *Daphnia magna* (see CA 9.2.4.1), testing of long-term and chronic toxicity to aquatic invertebrates is not considered necessary.

- Further, due to the low solubility of 24-Epibrassinolide in water and the readily uptake of free 24-Epibrassinolide by plants as well as the low application rate, no notable concentrations in surface water after application are expected and therefore further testing was not conducted.
- In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Overall RMS conclusion – Long-term toxicity to aquatic invertebrates:
No studies were submitted by the notifier to address the long-term effects on aquatic invertebrates. No explicit data was presented by the applicant regarding the natural background concentrations of 24-Epibrassinolide in water. In a public literature study by Hassett et al. (1977, reference KCA 8.2.5.4/0001, evaluated under point CA B.9.2.5.3) sterols were found in two North American lakes, with lake water sterol concentrations ranging from 0.7 – 3 µg/L. Although brassinosteroids are not explicitly mentioned, the referenced sterols are considered to have a close structural relation or represent even precursors of brassinosteroids and are therefore seen suitable to serve as a proxy to estimate the order of magnitude of 24-Epibrassinolide concentration in water. A worst case suface water PEC of 0.0232 µg a.s./L (FOCUS Step 2) was calculated in the fate section in Vol. 3 CP B.8. This supports the assumption that the natural exposure to 24-Epibrassinolide can be considered to be higher than the exposure following an application of the active substance in the form of a plant protection product. Thus the argumentation to waive the chronic aquatic invertebrates studies is considered acceptable due to the expected natural occurrence of brassinosteroids and other sterols in the environment and aquatic organisms. Further a low acute toxicity was demonstrated in *Daphnia magna* and a generally low water solubility of 3.8 mg/L (please refer to Vol. 3 CA Part B 2) is reported for the active substance. In conclusion adverse long-term effects to aquatic invertebrates posed by 24-Epibrassinolide are considered unlikely and the data requirement was sufficiently addressed.

B.9.2.5.1. Reproductive and development toxicity to an additional aquatic invertebrate species

Not required.

B.9.2.5.2. Development and emergence in Chironomus riparius

Not required.

B.9.2.5.3. Sediment dwelling organisms

The applicant submitted the following statement (in *italic*):
"Brassinosteroids – and other phytosterols – are naturally present in all environmental compartments including water-bodies and sediment (Hassett & Lee, 1977; Mudge et al. 1999). Therefore, and due to the low solubility of 24-Epibrassinolide in water and the readily uptake of free 24-Epibrassinolide by plants as well as the low application rate, no notable concentrations in surface water after application are expected. This is why also the concentrations in sediment from the application can be considered negligible and no testing on sediment dwelling organisms is considered necessary.
In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones."

Data point addressed:	CA B.9.2.5.3
Reference:	KCA 8.2.5.4/0001
Author(s) (year):	Hassett, J.P., Fred Lee, G., Lee, F.G. (1977)
Title:	STEROLS IN NATURAL WATER AND SEDIMENT
Published:	Yes
Published in:	Water Research, 1977, 11, 983-989
Test guideline used:	Not applicable
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional information**

Abstract provided by RMS:
„Sterols were detected in Lake Mendota, Wisconsin and Torch Lake, Michigan, water and sediment and in Lake Wingra, Wisconsin, sediment. These sterols included Compounds with gas Chromatography retention times equal to those for coprostanol, cholesterol, stigmasterol and β-sitosterol as well as other, unidentified compounds. In addition, cholestanol and perhaps other saturated sterols were present in Lake Mendota sediment. Sterol concentrations were 0.8 µg cholesterol/l and 3 µg β-sitosterol/l in Lake Mendota water and 0.7 µg cholesterol/1 and 2 µg β-sitosterol/l in Torch Lake water. Analysis of sediment sterols was probably not quantitative; however, the minimum concentration in Torch Lake sediment was about 3 ppm sterols on a dry weight basis. Both free sterols and sterol esters were present in Lake Mendota sediment, the sterols present as esters comprising roughly 10% of the total sterol content."

KCA 8.2.5.4/0001	**Comment RMS:** The referenced paper focusses on sterols found in two North American lakes, with lake water sterol concentrations ranging from 0.7 – 3 µg/L and lake water sediment sterol concentrations of ~ 3 mg/kg sediment dw. Several sterols were found, however although brassinosteroids are not explicitly mentioned the referenced sterols are considered to have a close structural relation or represent even precursors of brassinosteroids. Therefore the publication is seen to support the argument that sterols are found in lake water and sediment under natural conditions. This public literature paper is considered as additional information by RMS.

Data point addressed:	CA B.9.2.5.3
Reference:	KCA 8.2.5.4/0002
Author(s) (year):	Mudge, S.M., Joao A.F. Bebianno, M., East, J.A., Barreira, L.A. (1999)
Title:	STEROLS IN THE RIA FORMOSA LAGOON, PORTUGAL
Published:	Yes
Published in:	Water Research, 1999, 33 (4), 1038-1048
Test guideline used:	Not applicable
Deviations:	Not applicable
GLP:	No
Acceptability:	**No**

Abstract provided by RMS:
„Fifty nine surface sediment samples from the Ria Formosa lagoon were analysed for lipid biomarkers and 26 sterols were identified and quantified using a GC-MS technique. The total concentrations ranged from 0.1 to 27.8 µg/g [sediment] DW although this accounted for much less than 1% of the total organic carbon content. The principal sterols were cholesterol and β-sitosterol indicating marine fauna and terrestrial plants, respectively. Sewage markers (coprostanol and epi-coprostanol) were present around the known sewage discharge points at Faro, Tavira and Olhäo; the former two sites had untreated effluent whereas lhe latter site has partially treated discharges which was reflected in an increase in the epi-coprostanol/coprostanol ratio. Phytoplankton biomarkers (e.g. brassicasterol) were concentrated in the region near Armona, the major inlet to the lagoon from the Atlantic Ocean. Sterols indicative of other types of organic matter were also present but were restricted to selected regions. Multivariate Statistical techniques (PCA, PLS) were able to produce readily explainable diagrams which highlighted the contribution that each of the major sources, marine fauna, sewage, phytoplankton and terrestrial organic matter made to the sites of the lagoon."

KCA 8.2.5.4/0002	**Comment RMS:** The referenced paper focusses on sterols found in marine sediments. Several sterol groups are found, however, although 24-Epibrassinolide was not explicitly mentioned brassicasterol was found. The referenced sterols are considered to have a close structural relation or represent even precursors of brassinosteroids. Therefore it is seen to support the argument that brassinosteroids occur in marine sediment under natural conditions (in the range of 0.1 – 27.8 mg/kg sediment dw). However, since this study focusses on marine sediment concentrations it is unclear to which extent sediment dewelling organisms in marine and freshwater habitas differ in terms of sensitivity and species composition. Therefore this public literature paper is not considered reliable as additional information by RMS.

Overall RMS conclusion – Sediment dwelling organisms

No studies were submitted by the notifier to address the effects on sediment dwelling organisms. No explicit data was presented by the applicant regarding natural background concentrations of 24-Epibrassinolide in sediment. In the publication by Hassett et al. (1977) sterols were found in two North American lakes, with lake sediment sterol concentrations in the range of 3 mg/kg sediment dw. Although brassinosteroids are not explicitly mentioned, the referenced sterols are considered to have a close structural relation or represent even precursors of brassinosteroids and are therefore seen suitable to serve as a proxy to estimate the order of magnitude of 24-Epibrassinolide concentration in sediment. A worst case sediment PEC of 0.1632 µg a.s./kg sediment dw was calculated in the fate section in Vol. 3 CP B 8. This supports the assumption that the natural exposure to 24-Epibrassinolide can be considered to be higher than the exposure following an application of the active substance in the form of a plant protection product. Thus the argumentation to waive the studies on sediment dwelling organisms is considered acceptable due to the expected natural occurrence of brassinosteroids and other sterols in the environment and aquatic organisms. Further a low acute toxicity was demonstrated in *Daphnia magna* and a generally low water solubility of 3.8 mg/L (please refer to Vol. 3 CA Part B 2) is reported for the active substance. In conclusion adverse effects to sediment dwelling organisms posed by 24-Epibrassinolide are considered unlikely and the data requirement was sufficiently addressed.

B.9.2.6. Effects on algal growth

A waiver is requested for the performance of toxicity studies on algae because brassinosteroids are found in various algae species according to numerous public literature studies.

Data point addressed:	CA B.9.2.6
Reference:	KCA 8.2.6/0002
Author(s) (year):	Stirk, W.A., Balint, P., Tarkowska, D., Novak, O., Strnad, M., Oerdoeg, V., van Staden, J. (2013)
Title:	HORMONE PROFILES IN MICROALGAE: GIBBERELLINS AND BRASSINOSTEROIDS
Published:	Yes
Published in:	Plant Physiology and Biochemistry, 2013, 70, 348-353; doi: 10.1016/j.plaphy.2013.05.037
Test guideline used:	Not applicable
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional infromation**

Abstract (extracted by RMS):

Endogenous gibberellins and brassinosteroids were quantified in 24 axenic microalgae strains from the Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Charophyceae microalgae strains after 4 days in culture. This is the first report of endogenous gibberellins being successfully detected in microalgae. Between 18 and 20 gibberellins were quantified in all strains with concentrations ranging from 342.7 pg/mg DW in *Raplndocelis subcapitata* MACC 317-4746.1 pg/mg DW in *Scotiellopsis terrestris* MACC 44. Slower growing strains (S. *terrestris* MACC 44, *Gyoerffyana humicola* MACC 334, *Nautococcus mamillatus* MACC 716 and Chlorococcum *ellipsoideum* MACC 712) exhibited the highest gibberellin contents while lowest levels of gibberellins were found in faster growing strains (R. *subcapitata* MACC 317 and *Coelastrum excentrica* MACC 504). In all strains, the active gibberellin detected in the highest concentration was GA_6, the predominant intermediates were GA_{15} and GA_{53} and the main biosynthetic end products were GA_{13} and GA_{51}. Gibberellin profiles were similar in all strains except for the presence/absence of GA_{12} and GA_{12}ald. To date this is the second report of endogenous brassinosteroids in microalgae. Brassinosteroids were detected in all 24 strains with concentrations ranging from 117.3 pg/mg DW in *R. subcapitata* MACC 317-977.8 pg/mg DW In *Klebsormidium flaccidwn* MACC 692. Two brassinosteroids, brassinolide and castasterone were determined in all the strains. Generally, brassinolide occurred in higher concentrations than castasterone.

KCA 8.2.6/0002	**Comment RMS:** The referenced paper focusses on the occurrence on brassinosteroids and gibberellins in several microalgae species.

Table 3
Brassinosteroids (BRs) quantified in 24 microalgal strains analyzed after 4 days in culture. Results are shown as mean ± SD (n = 3).

Species	Strain	Brassinolide	Castasterone	Total BR content
	MACC	pg mg^{-1} DW		
Stigeoclonium nanum	790	168.7 ± 0.8	144.9 ± 12.1	313.6
Chlorococcum ellipsoideum	712	168.7 ± 14.5	105.7 ± 5.4	274.4
Gyoerffyana humicola	334	270.9 ± 32.8	201.1 ± 19.4	472.0
Monoraphidium contortum	700	284.9 ± 17.2	195.0 ± 4.1	479.9
Nautococcus mamillatus	716	115.8 ± 14.5	99.9 ± 12.2	215.7
Poloidion didymos	545	167.3 ± 15.6	172.8 ± 19.3	340.1
Protosiphon botryoides	32	100.6 ± 3.2	74.0 ± 15.8	174.6
Acutodesmus acuminatus	41	125.1 ± 7.8	105.5 ± 4.2	230.6
Acutodesmus incrassatulus	730	124.8 ± 12.6	92.6 ± 6.1	217.4
Desmodesmus armatus	59	125.1 ± 5.7	109.3 ± 0.2	234.4
Scotiellopsis terrestris	44	336.9 ± 40.0	235.9 ± 4.9	572.8
Raphidocelis subcapitata	317	58.6 ± 6.8	58.7 ± 7.6	117.3
Chlamydomonas reinhardtii	772	162.9 ± 4.2	153.8 ± 15.3	316.7
Protococcus viridis	219	211.6 ± 11.8	134.8 ± 8.3	346.4
Coelastrum microporum	51	199.2 ± 19.7	158.3 ± 18.6	357.5
Spongiochloris excentrica	504	131.2 ± 10.6	108.5 ± 1.8	239.7
Coccomyxa sp.	535	205.8 ± 3.9	177.1 ± 0.5	382.9
Chlorella pyrenoidosa	3	253.0 ± 14.7	158.0 ± 4.8	411.0
Chlorella vulgaris	755	193.3 ± 14.3	151.7 ± 4.9	345.0
Chlorella minutissima	361	306.5 ± 15.5	215.3 ± 19.4	521.8
Myrmecia bisecta	594	202.4 ± 44.5	164.3 ± 32.7	366.7
Stichococcus bacillaris	505	291.8 ± 7.0	242.7 ± 9.3	534.5
Ulothrix sp.	777	84.9 ± 5.3	74.2 ± 3.5	159.1
Klebsormidium flaccidum	692	548.7 ± 2.7	429.1 ± 30.8	977.8

The study supports that in the green algae *Chlorella vulgaris* brassinosteroid hormones in concentrations of 193.3 (± 14.3) µg/g dry weight were found. Overall, in the total of 24 microalgae species which were analysed the measured values of brassinolide ranged between 56.6 µg/g DW (*Raphidocelis. subcapitata*) and 548.7 µg/g DW (*Klebsormidium flaccidum*).

This public literature paper is considered as additional information by RMS, supporting the fact that brassinolide occurs naturally in algae tissue in relevant amounts.

Next to the study by Stirk *et al.* (2013) several papers on the effect of brassinolide have been published for *Chlorella vulgaris* and were submitted and briefly summarized by the applicant (in *italics*):

Bajguz (2009)[25] *found that brassinosteroids and brassinolide are naturally present in the green alga Chlorella vulgaris. By GC-SIM-MS, 0.07 ng/g brassinolide were identified as endogenous brassinolide in wild-type C. vulgaris. In addition, it is believed that endogenous brassinosteroids are required for normal development of C. vulgaris in light and brassinosteroids can be synthesised by C. vulgaris (**Bajguz & Asami, 2004**[26]).*

Bajguz (2011)[27] *reported the suppression of Chlorella vulgaris growth by cadmium, lead and copper stress and its restoration by endogenous brassinolide. It was found that brassinolide plays a positive role in the alleviation of heavy metal stress, as it reduced the accumulation of heavy metal stress on growth, prevented chlorophyll, monosaccarides and protein loss and increased phytochelatins content. Further, addition of brassinolide (10^{-8} M ethanolic solution) to the growth medium with or without heavy metals did not change the endogenous level of*

[25] KCA 8.2.6.1/0001: Bajguz, A. (2009): ISOLATION AND CHARACTERIZATION OF, BRASSINOSTEROIDS FROM ALGAL CULTURES OF CHLORELLA VULGARIS BEIJERINCK (TREBOUXIOPHYCEAE), Report No.: na (092-013), Journal of Plant Physiology, 2009, 166, 1946-1949; doi:10.1016/j.jplph.2009.05.003, Not GLP, published

[26] KCA 8.2.6.1/0002: Bajguz, A., Asami, T. (2004): EFFECTS OF BRASSINAZOLE, AN INHIBITOR OF BRASSINOSTEROID BIOSYNTHESIS, ON LIGHT- AND DARK-GROWN CHLORELLA VULGARIS, Report No.: na (092-101), Planta, 2004, 218, 869-877; DOI 10.1007/s00425-003-1170-9, Not GLP, published

[27] KCA 8.2.6.1/0004: Bajguz (2011): SUPPRESSION OF CHLORELLA VULGARIS GROWTH BY CADMIUM, LEAD, AND COPPER STRESS AND ITS RESTORATION BY ENDOGENOUS BRASSINOLIDE, Archives of Environmental Contamination and Toxicology, 2011, 60, 406-416; DOI 10.1007/s00244-010-9551-0, Not GLP, published

brassinolide in C. vulgaris. Even though it is not reported which brassinosteroid was used for the test, it is expected that 24-Epibrassinolide would have a similar effect on C. vulgaris.

Bajguz & Pitrowska-Niczyporuk (2014)[28] *studied the interactive effect of brassinosteroids and cytokinins on growth, chlorophyll, monosaccharide and protein content in the green alga Chlorella vulgaris, reporting a synergist relationship between brassinosteroids and cytokinis, as the effect of two hormones applied simultaneously exceeds the sum of each effect on algae growth and metabolic content. For 24-Epibrassinolide, data suggests an increase in growth at 10 nM (corresponding to 480 ng/L) compared to control (4.9*10^6 cells/ml compared to 2*10^6 cells/ml), an increase in protein content, chlorophyll and monosaccaride content. This might be due to an enhanced nutrient uptake. Further, the stimulatory effect of brassinosteroids was ranged with 24-Epibrassinolide as second active hormone after brassinolide, when used not in combination with cytokins. These results support the beneficial effect of 24-Epibrassinolide on algae and indicate that no toxicity from application of 24-Epibrassinolide is expected when applied according to GAP.*

All in all it can be concluded that no studies on the effect of 24-Epibrassinolide on algae growth are considered necessary as brassinosteroids are endogenous, synthesised by algae and have positive effects on algae in general. In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Further, the following paper about a diatom species was identified during EFSA-compliant literature search and was considered relevant for assessment on full-text level.

Data point addressed:	CA B.9.2.6
Reference:	KCA 8.2.6/0001
Author(s) (year):	Mekhalfi, M., Avilan, L., Lebrun, R., Botebol, H., Gontero, B. (2012)
Title:	CONSEQUENCES OF THE PRESENCE OF 24-EPIBRASSINOLIDE, ON CULTURES OF A DIATOM, ASTERIONELLA FORMOSA
Published:	Yes
Published in:	(Biochimie, 2012, 94, 1213-1220; doi: 10.1016/j.biochi.2012.02.011)
Test guideline used:	Not applicable
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional infromation**

Executive Summary (provided by the applicant):

In the research paper the addition of 24-Epibrassinolide (source Sigma Inc. USA) to culture media was found to stimulate the growth of *Asterionella formosa,* a freshwater diatom, as 24-Epibrassinolide stimulated key metabolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Further, the effect of 24-Epibrassinolide and illumination on enzyme activity in crude extracts and the effect of 24-Epibrassinolide on purified enzymes was determined.

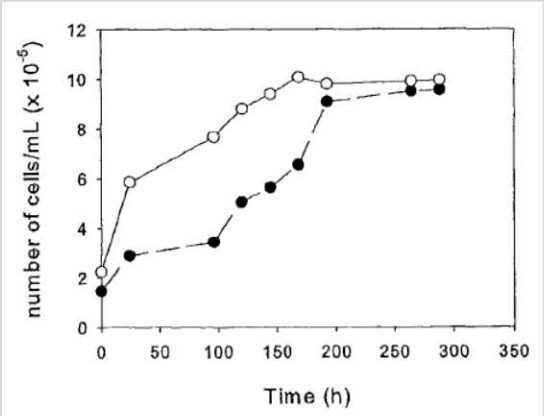
2 mg/L 24-Epibrassinolide was mixed with distilled sterilized water in the cultures of the diatom. The addition of 25 µL of the homogeneous suspension was added into 1 L of sterilized Diatom Medium, giving a final concentration of 0.1 µM (corresponding to 48 µg/L). The cell concentration was determined by measuring the optical density at 680 nm and also counting cells using a Lund chamber. In the presence of 24-Epibrassinolide cells entered the log phase sooner than those grown in the absence of 24-Epibrassinolide. The average growth rate (µ) from 8 cultures grown under normal conditions was (mean and standard deviation) 0.18 ± 0.02 day^{-1} corresponding to a generation time of 3.81 ± 0.41 days, and addition of 24-Epibrassinolide to cultures (n = 7) increased the growth rate to 0.25 ± 0.02 day^{-1} corresponding to a generation time of 2.73 ± 0.13 days. The difference between the growth rates of these two treatments was highly significant (Student's t-test, $P < 0.001$). However, the overall number of cells after 300 h was only slightly larger (10*10^{-5} cells/mL compared to and 9.5*10^{-5} cells/mL) for *A. formosa* grown in medium supplemented with 24-Epibrassinolide.

[28] KCA 8.2.6.1/0003: Bajguz, A., Piotrowska-Niczyporuk, A. (2014): INTERACTIVE EFFECT OF BRASSINOSTEROIDS AND CYTOKININS ON GROWTH, CHLOROPHYLL, MONOSACCHARIDE AND PROTEIN CONTENT IN THE GREEN ALGA CHLORELLA VULGARIS (TREBOUXIOPHYCEAE), Report No.: na (092-102), Plant Physiology and Biochemistry, 2014, 80, 176-183; doi: 10.1016/j.plaphy.2014.04.009, Not GLP, published

Discussion by the applicant:

The effects of 24-Epibrassinolide on purified enzymes as well as on crude extracts are not within the scope of this dossier, as they are scientific research results that give an indication on the possible mechanisms involved by adding 24-Epibrassinolide to *Asterionella formosa.* Therefore, only the *in vivo* influence, i.e. the growth rate stimulation will be discussed here.

As 24-Epibrassinolide enhanced the activity of GAPDH, a ubiquitous enzyme involved in the Calvin cycle, six fold, and other key enzymes involved in glycolysis (PFK), Krebs cycle (NAD-MDH) and oxidative pentose phosphate pathway (G6PDH) by the factor two, it is not surprising that a growth stimulation was observed.
Therefore, other naturally occurring algae species are likely to also profit from 24-Epibrassinolide, if it should be present in water in sufficient concentration (e.g. *Chlorella vulgaris*, see Bajguz 2011). However, 24-Epibrassinolide is ubiquitous, quickly absorbed by plants and therefore not expected to occur freely in water through the applications according to GAP. In addition, as overall number of cells was only slightly influenced by addition of 24-Epibrassinolide, it is likely that faster growth was due to increased nutrient uptake. Through competition between different algae species, such an effect might not occur under non-laboratory conditions. This leads to the conclusion that the effect on *Asterionella formosa* is expected to be minimal if occurring at all under non-laboratory conditions.

KCA 8.2.6/0001	**Comment RMS:** The referenced paper focusses on the effects of 24-Epibrassinolide on the diatom *Asterionella formosa*.  **Fig. 1.** *Asterionella formosa* growth rates. Cells from *A. formosa* grown in diatom medium (full circles) or in medium supplemented with 24-epibrassinolide at 0.1 µM (open circles) were counted as a function of time. The study reports that the addition of 48 µg/L 24-Epibrassinolide stimulated after exposure the growth of the diatom *A. formosa* but did not lead to a significant increased cell number compared to the standard medium at test end after 300 hours exposure (12.5 days). It appears that the test was conducted in a static test design, no analytical verification at test start and end is reported, thus the reliability is limited. Nonetheless, this public literature paper is considered as additional information by RMS, supporting that it is likely that no adverse effects on diatoms are induced by 24-Epibrassinolide in non-static conditions.

Overall RMS conclusion – Effects on algal growth:

No studies were submitted by the notifier to address the effects on algae. No explicit data was presented by the applicant regarding natural background concentrations of 24-Epibrassinolide in surface water. In the study by Stirk *et al.* (2013) 24 microalgae species were analysed and the measured values of brassinolide ranged between 56.6 µg/g DW (*Raphidocelis. subcapitata*) and 548.7 µg/g DW (*Klebsormidium flaccidum*). Although 24-Epibrassinolide is not explicitly mentioned, the referenced brassinolide are considered to have a close structural relation or represent even precursors of 24-Epibrassinolide and are therefore seen suitable to serve as a proxy to support that algae plant tissue naturally contains the active substance in relevant amounts.
In the experiment by Mekhalfi *et al.* (2012) nominally 48 µg 24-Epibrassinolide/L were added to the growth medium of diatoms, but the study did neither follow an OECD test guideline nor was it performed according to GLP standards. No analytical verification is reported at all, therefore the actual exposure over time in this static test design remains unlclear. However a worst case suface water PEC of 0.0232 µg a.s./L (FOCUS Step 2) was

calculated in the fate section in Vol. 3 CP B 8. This worst case surface water concentration is > 2000 times below the concentration used in the study by Mekhalfi *et al.* (2012). Further in a public literature study by Hassett et al. (1977, reference KCA 8.2.5.4/0001, evaluated under point CA B.9.2.5.3) sterols were found in two North American lakes, with lake water sterol concentrations ranging from 0.7 – 3 µg/L. Due to their structural similarity the referenced sterols (cholesterol and β-sitosterol) are considered suitable to serve as a proxy to estimate the order of magnitude of 24-Epibrassinolide concentration in natural surface water.
This supports the assumption that the natural exposure to 24-Epibrassinolide can be considered to be higher than the exposure following an application of the active substance in the form of a plant protection product. Thus the argumentation to waive the standard laboratory study with algae is considered acceptable due to the expected natural occurrence of 24-Epibrassinolide and other brassinosteroids in the environment and aquatic organisms. Further a generally low water solubility of 3.8 mg/L (please refer to Vol. 3 CA Part B 2) is reported for the active substance. In conclusion adverse effects to algae posed by 24-Epibrassinolide are considered unlikely and the data requirement was sufficiently addressed.
Although negative effects are considered unlikely, algae appear to be potentially susceptible organisms (due to possible effects on growth under static exposure laboratory conditions). Such potential effects under hazard lab study conditions can't be fully excluded since no studies are presented and the active substance is considered as not readily biodegradable (please refer to the RMS fate comment in Vol. 3 CA B8 under point 7.2.2.1). Therefore it is proposed to classify 24-Epibrassinolide according to Regulation (EU) 286/2011 within the "safety net" as hazard class "aquatic chronic 4 (H413)".

B.9.2.6.1. Effects on growth of green algae

Please refer to B.9.2.6.

B.9.2.6.2. Effects on growth of an additional algal species

Further testing is not considered necessary, as 24-Epibrassinolide is naturally occurring in several algae species. Please refer to B.9.2.6.

B.9.2.7. Effects on aquatic macrophytes

A waiver is requested for the performance of toxicity studies on aquatic macrophyts because brassinosteroids are found in various monocotyle and dicotyle plant species and thus is also considered a natural component of aquatic macrophytes. 24-Epibrassinolide has been found together with other brassionosteroides in sea bamboo *Ecklonia maxima*, used to produce the commercial seaweed extract "Kelpak", which is registered as a biostimulant e.g. in Europe (Stirk *et al.*, 2014).

As brassinosteroids and 24-Epibrassinolide naturally occur in aquatic macrophytes, no studies to address this point are considered necessary.

Data point addressed:	CA B.9.2.7
Reference:	KCA 8.2.7/0001
Author(s) (year):	Stirk, W.A., Tarkowska, D., Turecova, V., Strnad, M., van Staden, J. (2014)
Title:	ABSCISIC ACID, GIBBERELLINS AND BRASSINOSTEROIDS IN KELPAK®, A COMMERCIAL SEAWEED EXTRACT MADE FROM ECKLONIA MAXIMA
Published:	Yes
Published in:	Journal of Applied Phycology, 2014, 26, 561-567; DOI 10.1007/s10811-013-0062-z
Test guideline used:	Not applicable
Deviations:	Not applicable
GLP:	No
Acceptability:	**No**

Abstract (provided by RMS):
The seaweed extract Kelpak® made from the kelp *Ecklonia maxima* is registered as a biostimulant for use in agriculture. It elicits many beneficial responses including improved root and shoot growth, higher yields and greater resistance to abiotic and biotic stresses. Previously, cytokinins, auxins and polyamines were identified in Kelpak®. The aim of the present study was to quantify other groups of plant growth regulators (PGRs) — abscisic acid (ABA), gibberellins (GAs) and brassinosteroids — that may be present in *E. maxima* and Kelpak®. Kelpak® samples harvested between 2008 and 2010 and stored for up to 26 months were analysed using ultra Performance liquid chromatography tandem mass spectrometry. ABA levels were below the limits of detectionin *E. maxima* but were detected in low concentrations in Kelpak®, ranging from 0.31 to 20.70 pg/mL Kelpak®. Eighteen GAs were found in *E. maxima* and Kelpak with concentrations from 187.54 to 565.96 pg/mL Kelpak®. The biologically active GAs (GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7) comprised less than 3 % in Kelpak®. Although GA_{13} (a fmal product in the metabolic pathway) was present in low concentrations in *E. maxima,* very high concentrations were present in Kelpak®. The brassinosteroids brassinolide (BL) and castasterone (CS) were identified in *E. maxima* and Kelpak®. Concentrations varied with harvest and storage time, ranging from 384.72 to 793.23 pg BL/mL Kelpak® and 62.84 to 567.51 pg CS/mL Kelpak®. It is likely that this cocktail of natural PGRs present in Kelpak® may act individually or in concert and thus contribute to the ntmierous favourable physiological responses elicited by Kelpak® application to plants.

KCA 8.2.7/0001

Comment RMS:
The referenced paper focusses on the occurrence of natural plant growth regulators including brassinosteroids in a commercial seaweed extract. In the study brassinolide were found in the seaweed tissue of *E. maxima* (4.58 – 12.50 pg/mg dry weight) and in a commercial seaweed extract (20.7 – 829.2 pg/ml extract).

Table 3 Brassinosteroids quantified in *E. maxima* and the seaweed extract Kelpak®. Kelpak® was harvested over a 2-year period and stored for up to 26 months. The values of the two samples from each harvest are given (replicate 1; replicate 2)

Sample		Brassinolide	Castasterone	Total
pg mg^{-1} DW				
E. maxima	Stipe	11.76; 10.94	13.23; 7.94	25.0; 18.9
	Frond	4.58; 12.50	9.22; 16.42	13.8; 28.9
pg mL^{-1} Kelpak®				
Kelpak®	2 months	627.3; 522.6	486.3; 257.3	1116.5; 780.0
	8 months	20.7; 748.7	130.8; 648.3	151.5; 1397.1
	14 months	515.9; 600.2	437.6; 397.2	953.5; 997.4
	20 months	829.2; 757.2	467.1; 668.0	1296.3; 1425.2
	26 months	795.9; –	125.7; –	921.6; –

This public literature paper is considered as additional information by RMS, providing evidence that brassinolide are naturally occurring in aquatic macrophyts. However since this study focusses on marine macrophyts (seaweed is not a standard laboratory test species) it is unclear to which extent aquatic macrophyts in marine and limnological habitas differ in terms of sensitivity. Therefore this public literature paper is not considered reliable as additional information by RMS.

Overall RMS conclusion – Effects on aquatic macrophyts:
No studies were submitted by the notifier to address the effects on aquatic macrophyts. No explicit data was presented by the applicant regarding natural background concentrations of 24-Epibrassinolide in surface water. In public literature it is well documented, that brassinosteroids are considered essential for normal plant growth and development. Brassinosteroids, including 24-Epibrassinolide are naturally occurring, plant growth promoting molecules, present in higher plants and lower plants. Pollen and immature seeds contain the highest amount of brassinosteroids with a range of 1-100 µg/kg fresh weight, while shoots and leaves usually have lower amounts of 0.01 - 0.1 µg/kg fresh weight.[29]
The study by Stirk *et al.* (2013; reference KCA 8.2.6/0002, evaluated under point CA B.9.2.6) shows that aquatic microalgae tissue of several species naturally contain brassinolide in relevant amounts (56.6 µg/g dry weight in *Raphidocelis. subcapitata* and 548.7 µg/g dry weight in *Klebsormidium flaccidum*). Stirk *et al.* (2014) found

[29] KCA 8/0012: Bajguz, A. (2011): BRASSINOSTEROIDS – OCCURRENCE AND CHEMICAL STRUCTURES IN PLANTS. In: Hayat, S., Ahmad, A.: BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE. Report No.: na (092-146). Springer Verlag, 2011, Chapter 1, 1-27, DOI 10.1007/978-94-007-0189-2_1; ISBN: 978-94-007-0188-5. Not GLP, published

brassinolide in the seaweed tissue of *E. maxima* (4.58 – 12.50 ng/g dry weight). Therefore it is considered reasonable that also aquatic macrophyts naturally contain brassinosteroids (including 24-Epibrassinolide).
Further in a public literature study by Hassett et al. (1977, reference KCA 8.2.5.4/0001, evaluated under point CA B.9.2.5.3) sterols were found in two North American lakes, with lake water sterol concentrations ranging from 0.7 – 3 µg/L. Due to their structural similarity the referenced sterols (cholesterol and β-sitosterol) are considered suitable to serve as a proxy to estimate the order of magnitude of 24-Epibrassinolide concentration in natural surface water. A worst case suface water PEC of 0.0232 µg a.s./L (FOCUS Step 2) were calculated in the fate section in Vol. 3 CP B 8. This provides evidence that the natural exposure to 24-Epibrassinolide can be considered to be higher than the exposure following an application of the active substance in the form of a plant protection product. Thus the argumentation to waive the standard laboratory study with aquatic macrophyts is considered acceptable due to the expected natural occurrence of 24-Epibrassinolide and other brassinosteroids in the environment and aquatic organisms. Further a generally low water solubility of 3.8 mg/L (please refer to Vol. 3 CA Part B 2) is reported for the active substance. In conclusion adverse effects to aquatic macrophyts posed by 24-Epibrassinolide are considered unlikely and the data requirement was sufficiently addressed.
Although negative effects are considered unlikely, macrophyts appear to be together with algae potentially susceptible organisms (due to possible effects on growth under static exposure laboratory conditions). Such potential effects under hazard lab study conditions can't be fully excluded since no studies are presented and the active substance is considered as not readily biodegradable (please refer to the RMS fate comment in Vol. 3 CA B8 under point 7.2.2.1). Therefore it is proposed to classify 24-Epibrassinolide according to Regulation (EU) 286/2011 within the "safety net" as hazard class "aquatic chronic 4 (H413)".

B.9.2.8. Further testing on aquatic organisms

Not required.

B.9.3. EFFECTS ON ARTHROPODS

Most insects depend on plant steroids such as brassinosteroids as source of cholesterol, which is then converted to ecdysone, a hormone regulating gene expression e.g. for larval molting, adult leg morphogenesis and cuticle production (Thummel & Chory, 2002[30]). Brassinosteroids and 24-Epibrassinolide is therefore an essential part of arthropod's diet and arthropods are naturally exposed to these substances.
In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones. Therefore 24-Epibrassinolide is considered to have a low toxicity to non-target arthropods.

B.9.3.1. Effects on bees

24-Epibrassinolide is naturally found in various plants, pollen and honey. According to Ikekawa *et al.* (1988)[31], the highest concentration of 24-Epibrassinolide in bee pollen of *Vicia faba* was 5 µg/kg fresh weight and Khripach *et al.* (2013)[32] reported 7.4 ng/g 24-Epibrassinolide in honey.

[30] KCA 8.3/0001: Thummel, C.S., Chory, J. (2002): STEROID SIGNALING IN PLANTS AND INSECTS - COMMON THEMES, DIFFERENT PATHWAYS, Report No.: na (092-114), Genes & Development, 2002, 16, 3113-3129; DOI: 10.1101/gad.1042102, Not GLP, published

[31] KCA 8.3.1/0002: Ikekawa, N., Nishiyama, F., Fujimoto, Y. (1988): IDENTIFICATION OF 24-EPIBRASSINOLIDE IN BEE POLLEN OF THE BROAD BEAN, VICIA FABA L., Report No.: na (092-027), Chemical and Pharmaceutical Bulletin, 1988, 36 (1), 405-407, Not GLP, published

[32] KCA 8.3.1/0003: Khripach, V.A., Litvinovskaya, R.P., Kurtikova, A.L., Drach, S.V., Pryadko, A.G., Mirantsova, T.V., Baranovskiy, A.V. (2013): ENZYME IMMUNOASSAY OF THE CONTENT OF ENDOGENOUS BRASSINOSTEROIDS IN PHYTOGENIC FOOD PRODUCTS, Report No.: na (092-030), National Academy of Sciences of Belarus, 2013, 57 (2), 63-69, Not GLP, published

The following paper by Chuda-Mickiewicz *et al.* (2009) about queen bees (Carnolian breed *Apis m. carnica*) was identified during EFSA-compliant literature search and was considered relevant for assessment on full-text level:

Data point addressed:	CA B.9.3.1
Reference:	KCA 8.3.1/0001
Author(s) (year):	Chuda-Mickiewicz, B., Prabucki, J., Samborski, J., Rostecki, P. (2009)
Title:	THE ROLE OF PHYTOHORMONES IN INSTRUMENTAL INSEMINATION OF QUEEN BEES
Published:	Yes
Published in:	Journal of Apicultural Science, 2009, 53 (2), 91-96
Test guideline used:	Not applicable
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional infromation**

Summary (provided by the applicant):
In total, 164 queen bees were inseminated instrumentally, including 122 ones in mating nuclei being fed with phytohormones-supplemented sugar syrup (cytokinin and epibrassinolide) before and/or after instrumental insemination with 8 µl of semen. It was found that administration of phytohormones to queen bees did not have an effect on insemination efficiency or shortening of the time for onset of oviposition.

Discussion by the applicant:
Feeding treatment with 25 mg cytokinin and 0.12 mg epibrassinolide per litre syrup either before and/or after insemination had no effect on insemination efficiency or onset of oviposition. Observed mortality in control group was similar to that of treated groups. Further, no other effects (e.g. sublethal or behavioural effects) for treated bee queens were reported. It is unclear, if 24-Epibrassinolide was used in the study; however, similar effects for 24-Epibrassinolide can be expected. This study is therefore considered valid to provide further evidence on the non-toxic properties of 24-Epibrassinolide to bees.

KCA 8.3.1/0001	**Comment RMS:** The referenced paper focusses on the effects on queen bees (Carnolian breed *Apis m. carnica*) fed with sugar syrup supplemented with phytohormones (including 0.12 mg epibrassinolide/L syrup). There were 4 treatment groups: 1: Feeding queens with phytohormone supplemented syrup for 3 days before insemination. 2: Feeding queens with phytohormone supplemented syrup for 3 days after insemination. 3: Feeding queens with phytohormone supplemented syrup for 2 days before and for 2 days after insemination. 4: Control - Feeding queens with syrup without phytohormone supplemented for 2 days after insemination. The study did not follow a standardized protocol and no explicit information regarding actual syrup uptake or housing conditions is presented. The study finds that the uptake of the phytohormones did not have an effect on the egg laying performance of the queens. This public literature study is considered as additional information by RMS, providing evidence that adverse effects on queen bee egg laying behaviour are unlikely induced by 24-Epibrassinolide.

B.9.3.1.1. Acute toxicity to bees

B.9.3.1.1.1. Acute oral toxicity

The following bee acute oral study was conducted in support of the assessment and has not been previously evaluated.

Data point addressed:	CA B.9.3.1.1.1
Reference:	KCA 8.3.1.1.1/0001
Author(s) (year):	Bharathiraja, K. (2017a)

Title:	Acute Oral Toxicity Study in Honey bees (Apis mellifera) with 24-Epibrassinolide (TGAI)
Laboratory report / project Number (Doc No.):	6125
Testing facility:	RCC Laboratories India Private Limited
Published:	No
Test guideline used:	OECD No. 213
Deviations:	None
GLP:	Yes
Acceptability:	**Yes**

Executive Summary

An acute oral toxicity study in honey bees, in accordance with OECD-213 was conducted with 24-Epibrassinolide (TGAI) in a limit test design. Honey bees were orally treated with 100 µg a.s./bee in diet (50% w/v sucrose solution), considering the active substance of the test item 24-Epibrassinolide (TGAI). Five replicates were maintained and each test cage contained ten honey bees. Control and treatment group of bees were maintained. The test item was formulated in 50% w/v sucrose solution. Control group was given only 50% w/v sucrose solution.

The bees were acclimatized prior to the treatment for approximately 2 hours under the test conditions and they were kept under starvation for 2 hours before the test item administration. After treatment on day 0, the bees were observed for mortality and toxicity signs at 4th hour and then after at 24 and 48 hours. Concurrent control group was maintained under the test conditions.

At the end of 4, 24 and 48 hours, no mortality and toxicity signs were observed in bees of control and bees treated with 100 µg a.s./bee concentration.

On completion of test, all live bees were anaesthetized with carbon-dioxide and disposed off along with dead bees in toxic waste disposal.

Reference study with a toxic reference standard Dimethoate was conducted along with the limit test and the LD_{50} of Dimethoate observed for a period of 24 hour was calculated to be 0.20 µg a.s/bee which met the specified range (0.10 – 0.35 µg a.s/bee).

The endpoint of this study was therefore determined to be:

LD50 > 92.2034 µg a.s./bee (actually consumed dose)

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material:

Test Material:	24-Epibrassinolide (TGAI)
Description:	Odorless Oyster White Solid Powder
Lot/Batch #	002-20150112
Purity:	91.2 % w/w
Stability of test compound:	The test item is thermally stable for 14 days at 54 ± 2 °C

2. Vehicle and/or positive control:

Vehicle:	Test item was formulated in 50% w/v sucrose solution
Positive control:	0.03, 0.08, 0.24 and 0.66 µg Dimethoate/bee

3. Test animals

Species:	Honey bees (*Apis mellifera*)
Source:	In-house Apiary, RCC Laboratories India Private Limited (Supplied from Apiary, Bangalore rural (Dist) Pin 561 203)
Age:	Young Adult Worker bees
Weight at dosing:	Not reported
Acclimation period:	2 h before the test
Environmental conditions:	
Temperature:	23.8 – 25.7° C
Humidity:	54 – 65 %
Photoperiod:	Complete darkness except during the observation period

A. STUDY DESIGN AND METHODS

In-life dates 27 April, 2016 – 17 July, 2016

The acute oral toxicity of 24-Epibrassinolide (TGAI) on young adult worker bees was determined by offering test item treated sucrose solution. The study included 5 treatment groups (nominal 1, 10, 25, 50 and 100 µg/bee), a control and 4 reference item groups with 2 replicates each for range finding.
For limit test, a concentration of 100 µg/bee in 5 replicates was used. A single replicate contained a group of 10 bees. Mortality was assessed at 4 hours, 24 and 48 h after application. Dimethoate was used as reference item. Young adult worker bees were collected in the bee cages in the morning of use before the test and kept under test conditions for approximately 2 hours. The bee cages were randomly placed and only healthy bees were used for the test. Ten bees were placed in the test cage. For the test item exposure, the required concentration of test item was formulated in 50% w/v sucrose solution and fed to the honey bees in 1.5 ml eppendorf tube which has a hole at the side bottom. Each test group of bees was provided with 200 µl of 50 % w/v sucrose solution, containing the test item at the appropriate concentration. The sucrose solution with test item was provided approximately for a period of 4 hours, then all the treated group of bees were provided with only sucrose solution (*ad libitum*) without test item for a period of 48 hour.

II. RESULTS AND DISCUSSION

Results of the reference item toxicity test

The oral LD_{50} (24 hour) of the toxic reference standard – Dimethoate was calculated to be 0.20 µg a.s/bee with a lower confidence limits of 0.16 µg a.s/bee and upper confidence limits of 0.25 µg a.s/bee which met the specified range (0.10 – 0.35 µg a.s/bee) as stated in the OECD Guidelines 213/214. Therefore, the test system was sensitive.

Validity criteria

The study was considered to be valid since the following criteria were met:

- No mortality was observed in the control until completion of the test
- LD_{50} of the toxic reference standard, Dimethoate was calculated to be 0.2 µg a.s./bee which met the specified range (0.1 - 0.35 µg a.s./bee).

Oral toxicity test

In the range finding experiment, bees were treated with a series of concentrations of test item 1, 10, 25, 50, 100 µg/bee and 100 µg test item/bee in diet (50% w/v sucrose solution) and concurrent control group was also maintained. At the end of 4, 24 and 48 hours, no mortality and toxicity signs were observed in bees of control group and bees treated with 1, 10, 25, 50, 100 µg/bee and 100 µg test item/bee concentrations (see Table 9.3.1-1).

Based on the results of the range finding experiment, a limit test was conducted in which the bees were orally treated with 50% w/v sucrose solution containing the test item at 100 µg a.s/bee concentration. At the end of 4, 24 and 48 hours, no mortality and toxicity signs were observed in control and bees treated with 100 µg a.s/bee (109.66 µg test item/bee) concentration (see Table 9.3.1-2).

Table 9.3.1-1: Mortality of bees in the oral toxicity test (range-finder)

Treatment Group		**Cumulative Mortality [1]** **Time after Application**			
Nominal dose [µg/bee]	**Actually consumed dose [µg/bee]**	**4 h**	**24 h**	**48 h**	**Overall mortality [%]**
Test item					
0 (control)	0 (control)	0	0	0	0
1	0.9364	0	0	0	0
10	9.2797	0	0	0	0
25	23.1462	0	0	0	0
50	45.6568	0	0	0	0
100	89.6186	0	0	0	0

[1] values in parentheses represent sub-lethal effects (L = lethargic, E = loss of equilibrium)

Table 9.3.1-2: Mortality of bees in the oral toxicity test (limit test)

Treatment Group		**Cumulative Mortality [1]** **Time after Application**			
Nominal dose [µg/bee]	**Actually consumed dose [µg/bee]**	**4 h**	**24 h**	**48 h**	**Overall mortality [%]**

Test item					
0 (control)	0 (control)	0	0	0	0
100	92.2034	0	0	0	0
Reference item					
0.03		0	0	0	0
0.08		0	2 (L)	5 (L)	17%
0.24		5 (L)	17 (L)	18 (L)	60%
0.66		17 (L)	30	30	100%

[1] values in parentheses represent sub-lethal effects (L = lethargic, E = loss of equilibrium)

III. CONCLUSIONS

Honey bees (*Apis mellifera*) were exposed to 24-Epibrassinolide (TGAI) in an acute oral toxicity test at the limit concentration of nominal 100 µg a.s./bee. Based on the test results, the acute oral LD_{50} of 24-Epibrassinolide (TGAI) observed for a period of 48 hour was found to be greater than nominal 100 µg a.s/bee concentration (actually consumed dose 92.2 µg a.s./bee). The NOEC and LOEC were therefore determined to be nominal 100 µg a.s/bee and >100 µg a.s/bee.

KCA 8.3.1.1.1/0001	**Comment RMS:** The study is relevant and reliable. The validity criteria of OECD TG 213 (1998) are met. No deviations occurred. The endpoint is confirmed by RMS. **LD_{50} > 92.2 µg a.s./bee (actually consumed dose)**

B.9.3.1.1.2. Acute contact toxicity

The following bee acute contact study was conducted in support of the assessment and has not been previously evaluated.

Data point addressed:	CA B.9.3.1.1.2
Reference:	KCA 8.3.1.1.2/0001
Author(s) (year):	Bharathiraja, K. (2017b)
Title:	Acute Contact Toxicity Study in Honey bees (*Apis mellifera*) with 24-Epibrassinolide (TGAI)
Laboratory report / project Number (Doc No.):	6124
Testing facility:	RCC Laboratories India Private Limited
Published:	No
Test guideline used:	OECD No. 214
Deviations:	The study was performed at a concentration of 10 µg/bee instead of 100 µg a.s/bee because of solubility of test item. No effect on study integrity.
GLP:	Yes
Acceptability:	**Yes**

Executive Summary

An acute contact toxicity study in honey bees was conducted in accordance with OECD-214 for 24-Epibrassinolide (TGAI) in a limit test design. Bees were treated with maximum solubility limit of 10 µg/bee concentration, considering maximum solubility of the test item. Five replicates were maintained and each replicate contained ten honey bees. The test item was dissolved in acetone and applied at 1µl per bee.

The bees were acclimatized prior to the treatment for approximately 2 hours in the test conditions. After treatment on day 0, the bees were observed for mortality and toxicity signs approximately at 4th hour and then after 24 and 48 hours. Concurrent control and solvent control group were maintained in the test conditions.

At the end of 4, 24 and 48 hour no mortality and toxicity signs were observed in control groups and bees treated with 10 µg/bee concentration.

On completion of test, all live bees were anaesthetized with carbon-dioxide and disposed off along with dead bees in toxic waste disposal.

Reference study with a toxic reference standard Dimethoate was conducted along with the limit test and the LD_{50} observed for a period of 24 hour was found to be 0.21 µg/bee which met the specified range (0.10 – 0.30 µg a.s./bee).

The endpoint of this study was therefore determined to be:

LD50 > 10 µg a.s./bee (solubility limit)

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material:

Test Material:	24-Epibrassinolide (TGAI)
Description:	Odorless Oyster White Solid Powder
Lot/Batch #	002-20150112
Purity:	91.2 % w/w
Stability of test compound:	The test item is thermally stable for 14 days at 54 ± 2 °C

2. Vehicle and/or positive control:

Vehicle:	Aceton
Positive control:	0.03, 0.08, 0.24 and 0.66 µg Dimethoate/bee

3. Test animals

Species:	Honey bees (*Apis mellifera*)
Source:	In house Apiary, RCC Laboratories India Private Limited (Supplied from Apiary, Bangalore rural (Dist) Pin 561 203)
Age:	Young Adult Worker bees
Weight at dosing:	Not reported
Acclimation period:	Approximately 2 hours
Environmental conditions:	
Temperature:	23.8 – 25.7°C
Humidity:	54 – 65 %
Photoperiod:	Complete darkness except during the observations

A. STUDY DESIGN AND METHODS

In-life dates 27 April, 2016 – 17 July, 2016

The acute contact toxicity of 24-Epibrassinolide (TGAI) on worker bees was determined by topical application.
To determine maximum solubility of the test compound for the application in the test, 100 mg of the test item was made up to 10 ml with acetone in a 10 ml standard flask. After ultrasonication for 1 minute, the test item was completely soluble. The resulting stock concentration prepared was 10 µg/µl.
Another 100 mg of the test item was made up to 5 ml with acetone in a 5 ml standard flask, corresponding to 20 µg/µL. The test item was not soluble even, after 10 minutes of ultrasonication.
Therefore, solubility of the test item was determined to be, 10 µg/µl for dose application in the study.

The study included 5 treatment groups (2, 4, 6, 8 and 10 µg/bee), a negative control, solvent control with 2 replicates each and 4 reference item groups (0.03, 0.08, 0.24 and 0.66 µg a.s./bee) with 3 replicates each. A single replicate contained a group of 10 bees. Mortality was assessed at 4 hours and 24 and 48 h after application. Dimethoate was used as reference item. Young, adult worker bees were collected in the bee cages in the morning and kept under test conditions on the dosing day for approximately 2 hours. The collected bees were anaesthetized with carbon dioxide before the application of the test item. Only healthy bees were used for the test. Anaesthetized bees were individually treated by topical application using hand microapplicator (Burkard Scientific). The bees were randomly assigned to the test concentration and control groups. A volume of 1 µl of solution containing the test item at the suitable concentration was applied with a hand microapplicator to the dorsal side of the thorax of each bee. After application, ten bees were allocated to each replicate of respective test cage. Treatment and observations were conducted under light, but during the conduct of experiment complete darkness was maintained. After test item application, 50% w/v sucrose solution was provided *ad libitum*.

II. RESULTS AND DISCUSSION

Toxicity of the reference item

Contact LD_{50} (24hour) of the toxic reference standard – Dimethoate is 0.21 µg a.s./bee with a lower confidence limits of 0.17 µg a.s./bee and upper confidence limits of 0.27 µg a.s./bee which met the specified range (0.10 – 0.30 µg a.s./bee). This corresponds to the specified range for the contact 24 h-LD_{50} of 0.1 – 0.3 µg a.s./bee as stated in the OECD Guideline 214 and showed that the test system was sensitive.

Validity criteria

The study was considered valid since the following criteria were met:

- No mortality was observed in control groups until the completion of the test
- LD_{50} of the toxic reference standard, Dimethoate is 0.21 µg a.s./bee, which met the specified range (0.1-0.3 µg/a.s./bee)

Contact toxicity test

In the range finding experiment, bees were treated with a series of concentrations of 2, 4, 6, 8 and 10 µg a.s./bee of the test item. At the end of 4, 24 and 48 hour no mortality and toxicity signs were observed in control groups and bees treated with 2, 4, 6, 8 and 10 µg/bee concentration (see Table 9.3.1-3).

Based on the range finding experiment, a limit test was conducted with 10 µg a.s./bee concentration, considering maximum solubility of the test item and 1 µl of the test item was applied on the thorax of the bee using hand microapplicator and the bees were provided with 50% w/v sucrose solution during the conduct of the study. At the end of 4, 24 and 48 hour no mortality and toxicity signs were observed in control groups and bees treated with 10 µg/bee concentration (see Table 9.3.1-4)

Table 9.3.1-3: Mortality of bees in the contact toxicity test (rang-finder)

Treatment Group	**Cumulative Mortality [1]** **Time after Application**			
Nominal dose [µg/bee]	**4 h**	**24 h**	**48 h**	**Overall mortality [%]**
Test item				
Control	0	0	0	0
Solven control	0	0	0	0
2	0	0	0	0
4	0	0	0	0
6	0	0	0	0
8	0	0	0	0
10	0	0	0	0

[1] values in parentheses represent sub-lethal effects (L = lethargic, E = loss of equilibrium)

Table 9.3.1-4: Mortality of bees in the contact toxicity test (limit test)

Treatment Group	**Cumulative Mortality [1]** **Time after Application**			
Nominal dose [µg/bee]	**4 h**	**24 h**	**48 h**	**Overall mortality [%]**
Test item				
Control	0	0	0	0
Solven control	0	0	0	0
10	0	0	0	0
Reference item				
0.03	0	0	0	0
0.08	0	3 (L)	5 (L)	17%
0.24	5 (L)	14 (L)	15 (L)	50%
0.66	18 (L)	30	30	100%

[1] values in parentheses represent sub-lethal effects (L = lethargic, E = loss of equilibrium)

III. CONCLUSIONS

Honey bees (*Apis mellifera*) were exposed to 24-Epibrassinolide (TGAI) in an acute contact toxicity test at the solubility limit of 10 µg/bee. Based on the test results, the acute contact LD_{50} of 24-Epibrassinolide (TGAI) observed for a period of 48 hour was found to be greater than 10 µg/bee concentration. The NOEC and LOEC were therefore determined to be greater than 10 µg/bee and > 10 µg/bee.

KCA 8.3.1.1.2/0001	**Comment RMS:** The study is relevant and reliable. The validity criteria of OECD TG 214 (1998) are met. No major deviations occurred. The endpoint is confirmed by RMS. **LD_{50} > 10 µg a.s./bee (solubility limit)**

Overall RMS conclusion – Acute toxicity to bees:
Table 9.3.1-5 summarises the results of all available acute honey bee toxicity studies conducted with 24-Epibrassinolide.

Table 9.3.1-5: Acute oral and contact toxicity of 24-Epibrassinolide to bees

	48 h LD_{50} [µg a.s./bee]	
Species	**Acute oral toxicity**	**Acute contact toxicity**
Apis mellifera	> 92.2 (actual consumed dose) Study: Bharathiraja, K. (2017a)	> 10 (solubility limit) Study: Bharathiraja, K. (2017b)

B.9.3.1.2. Chronic toxicity to bees

A waiver is requestet by the applicant for the performance of chronic toxicity studies with bees due to the ubiquitous occurrence of 24-Epibrassinolide and other brassinosteroids in plants and pollen, the natural exposure of bees to the substance, as well as the low acute oral and contact toxicity for bees. In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Comment RMS:
The waiver is considered acceptable in the light of weight of evidence. A low acute toxicity (zero mortality) was demonstrated in the standard laboratory bee studies and public literature supports that 24-Epibrassinolide is naturally found in various plants, pollen and honey. It is reasonable that bees are constantly exposed to brassinosteroids via food consumption. As referenced above in Ikekawa *et al.* (1988) the highest concentration of 24-Epibrassinolide in bee pollen of *Vicia faba* was 5 µg/kg fresh weight and Khripach *et al.* (2013) reported 7.4 ng/g 24-Epibrassinolide in honey. The public literature by Chuda-Mickiewicz *et al.* (2009, reference KCA 8.3.1/0001, evaluated above under point B.9.3.1) assessed the effects of queen bees (Carnolian breed *Apis m. carnica*) fed with sugar syrup supplemented with phytohormones (including 0.12 mg epibrassinolide/L syrup). Queen bees fed 2 days before and for 2 days after insemination with the supplemented syrup did not show effects on egg laying behaviour or mortality. Although the study did not follow a standardized protocol and no explicit information regarding actual syrup uptake or housing conditions are presented the paper is still considered to support that negative effects posed by 24-Epibrassinolide are rather unlikely.

Thus adverse effects posed by 24-Epibrassinolide are considered unlikely and the data requirement is considered sufficiently addressed.

B.9.3.1.3. Effects on honeybee development and other honeybee life stages

Please refer to B.9.3.1.2.

B.9.3.1.4. Sublethal effects

Please refer to B.9.3.1.2.

B.9.3.2. Effects on non-target arthropods other than bees

A waiver is requested by the applicant for the performance of the standard effect studies with non-target arthropds other than bees because due to the ubiquitous occurrence of 24-Epibrassinolide and other brassinosteroids in plants and plant parts, non-target arthropods are naturally exposed to the substance through feeding on plant sap, nectar or other plant-sucking insects. Because of this natural exposure as well as no indication in public literature that 24-Epibrassinolide has an effect on non-target arthropods, no studies are considered necessary.

In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Comment RMS: No studies were submitted by the notifier to address the effects of the active substance 24-Epibrassionolide on non-target arthropods other than bees. The waiver for studies with the active substance is accepted because on the one hand public literature shows the natural occurrence of brassinosteroids in plants and thus supports a natural exposure. On the other hand the applicant submitted standard laboratory studies with *A. rhopalosiphi* and *T. pyri* for the representative formulation "Sunergist" to fulfil the product data requirement (please refer to Vol. 3 CP B9 9.5.2).

B.9.3.2.1. Effects on *Aphidius rhopalosiphi*

Please refer to B.9.3.2.

B.9.3.2.2. Effects on *Typhlodromus pyri*

Please refer to B.9.3.2.

B.9.4. EFFECTS ON NON-TARGET SOIL MESO- AND MACROFAUNA

Due to the ubiquitous occurrence of 24-Epibrassinolide and other brassinosteroids in plants and plant material, non-target meso- and macrofauna such as earthworms are naturally exposed to plant hormones. Free 24-Epibrassinolide in soil will be absorbed by plants or degraded rapidly (please refer to Vol. 3 CA B 8) and exposure of soil organisms will therefore be minimal, if existing at all.
Further, Aremu *et al.* (2015) studied the concentration of brassinosteroid and other phytohormones in different vermicompost leachates, and confirmed that brassinosteroids are present in the organic matter of soil. The total brassinosteroid content measured was between 1.40 ng/L leachate (fg/mL) and 3248.22 ng/L leachate (fg/mL).
According to Badri & Vivanco (2009)[33] non target soil organisms are further exposed to the brassinosteroid precursors, campesterol, sitosterol, and stigmasterol. These precursors are known root exudates and are involved in the mediation of interactions in the rhizosphere, which includes the symbiotic associations with beneficial microbes, such as mycorrhizae, rhizobia, and plant growth-promoting rhizobacteria.
In addition, as brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

B.9.4.1. Earthworm – sub-lethal effects

A waiver is requested by the applicant for the performance of the standard effect studies with earthworms because they are naturally exposed to brassinosteroid-containing plant materials such as pollen or other plant parts and thus there is a constant natural intake of brassinosteroids via earthworm feed. As shown by Aremu *et al.* (2015), vermicompost leachates contain brassinosteroids, which supports that brassinosteroids can be considered to naturally pass through the gut of earthworms. No negative effects following such an exposure are referenced in public literature. Due to the fast uptake of brassinosteroids by plants in case of brassinosteroids coming in contact with soil and the rapid degradation (please refer to Vol. 3 CA B 8), no effects from the use of 24-Epibrassinolide according to GAP are expected on earthworms. Therefore, no effect studies were conducted.

[33] KCA 8.4/0002: Badri, D.V., Vivanco, J.M. (2009): REGULATION AND FUNCTION OF ROOT EXUDATES, Report No.: na (092-012), Plant, Cell and Environment, 2009, 32, 666-681; doi: 10.1111/j.1365-3040.2009.01926.x, Not GLP, published

Data point addressed:	CA B.9.4.1
Reference:	KCA 8.4.1/0001
Author(s) (year):	Aremu, A.O., Stirk, W.A., Kulkarni, M.G., Tarkowska, D., Tureckova, V., Gruz, J., Subrtova, M., Pencik, A., Novak, O., Dolezal, K., Strnad, M., Van Staden, J. (2015)
Title:	EVIDENCE OF PHYTOHORMONES AND PHENOLIC ACIDS VARIABILITY IN GARDEN-WASTE-DERIVED VERMICOMPOST LEACHATE, A WELL-KNOWN PLANT GROWTH STIMULANT
Published:	Yes
Published in:	Plant Growth Regulation, 2015, 75 (2), 483-492
Test guideline used:	None
Deviations:	Not applicable
GLP:	No
Acceptability:	**No**

Abstract provided by RMS:

Cytokinins, auxins, abscisic acid, gibberellins (GAs) and brassinosteroids (BRs) as well as the phenolic acid content in three batches of vermicompost leachate (VCL) were quantified using ultra high performance liquid chromatography-tandem mass spectrometry. N^6-isopente-nyladenine formed the major (60 %) proportion of the CK content while dihydrozeatin had the lowest (<0.02 %) concentration. Indole-3-acetic acid ranged from approximately 0.55-0.77 pmol/mL. A total of 18 GAs including bioactive forms and metabolic end products were observed in the VCL samples. Cathasterone had the highest (2,500-3,200 fg/mL) concentration while brassinolide was the lowest (1-5 fg/mL) abundant BRs found. Phenolic acids quantified were protocatechuic acid (3-3.6 µg/mL), p-hydroxybenzoic acid (2.5-2.8 µg/mL), p-coumaric acid (1-1.7 µg/mL) and ferulic acid (0-4 µg/mL). These results provide an indication of the rich diversity in natural PGRs and phytochemicals in VCL which may inevitably contribute to the numerous favorable physiological responses elicited by VCL application to plants.

KCA 8.4.1/0001	**Comment RMS:** The referenced paper focusses on the quantification of phytohormones in commercial vermicompost leachate. No details regarding the worm-content and worm species in the vermicompost are available. The study shows that brassinosteroids occur in the analysed vermicompost leachate (brassinolide between 1.40 – 5.29 fg/mL, other brassinosteroids range between 35.24 – 3248.22 fg/mL). However due to lacking details regarding the actual effects on earthworms (e.g. mortality, body weight change, reproduction) and the concentration given in mL leachate (instead of mg/kg compost) this study is not considered suitable to demonstrate sufficiently that no adverse effects on earthworms induced by 24-Epibrassinolide are to be expected. Thus this public literature study is not considered relevant or reliable as additional information by RMS.

Overall RMS conclusion – Sub-lethal effects on earthworms:

No laboratory studies were submitted by the notifier to address the effects on earthworms. No explicit data was presented by the applicant regarding natural background concentrations of 24-Epibrassinolide in soil. The public literature study by Aremu *et al.* (2015) is not considered acceptable by RMS. It provides evidence that vermicompost leachate contains brassinosteroids, however due to lacking details regarding effects on earthworms (e.g. mortality, body weight change, reproduction) and the concentration of brassinosteroids in the compost (given in mg/kg compost) this study is not considered reliable to demonstrate that no adverse effects on earthworms induced by 24-Epibrassinolide are to be expected.

However, in the Fate section (Vol. 3 CA B.8.1.1.1) it is referenced, that Heumann *et al.* (2011)[34] studied the phytosterol content in soil samples. The samples were taken from different soil types such as podzoles, gleysoles cambisols and intermediates. In the measured soil samples the overall sterol concentrations ranged between 100 and 3600 mg/kg soil, the concentrations of the brassinosteroid precursor β-sitosterol ranged from 1 – 100 mg/kg

[34] KCA 7.1.1.1/0005: Heumann, S., Schlichting, A., Böttcher, J., Leinweber, P. (2011): Sterols in soil organic matter in relation to nitrogen mineralization in sandy arable soils, J. Plant Nutr. Soil Sci., 2011, 174, 576-586; doi: 10.1002/jpln.200900273, Not GLP, published

soil. Due to the relative structural similarity the referenced sterols are considered suitable to serve as a proxy to estimate the order of magnitude of 24-Epibrassinolide concentration in soil.
Worst case PECsoil values were calculated in the fate section (please refer to Vol. 3 CP B 8). Following the maximum application rate of 3 x 0.05 g a.s./ha in grapes the resulting PECsoil is 0.0002 mg a.s./kg soil (with worst case assuptions of 0 % crop interception and a $DT_{50\ soil}$ of 69.02 days) and covers the GAP of all other uses. This maximum worst case PECsoil of 0.0002 mg a.s./kg soil is around ~ 5000 times below the lowest reported soil sterol concentrations (i.e. 1 mg β-sitosterol/kg soil). This provides evidence that the natural exposure to brassinosteroids (including 24-Epibrassinolide) can be considered to be higher than the exposure following an application of the active substance in the form of a plant protection product.
Moreover free phytohormones such as 24-Epibrassinolide in soil are considered to be readily taken up by plants via roots.[35] Thus, it is to be expected that in case brassinosteroids occur freely in soil, e.g. if brassinosteroids are released by degradation of organic plant matter or during the use of brassinosteroid-containing plant protection products, brassinosteroids are taken up by roots and subsequently metabolised by plants.

In conclusion the waiving of the sub-lethal effect study with earthworms is considered acceptable. Adverse effects posed by 24-Epibrassinolide are considered unlikely and the data requirement is considered sufficiently addressed.

B.9.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)

A waiver is requested by the applicant for the performance of the standard effect studies with soil organisms (other than earthworms) because they are naturally exposed to brassinosteroid-containing plant materials such as pollen or other plant parts and thus there is a constant natural intake of brassinosteroids via feed (please refer to B.9.4.2). Moreover no adverse effects from the use of 24-Epibrassinolide are expected on non-target meso- and macrofauna in regard of the low toxicity demonstrated in non-target arthropods (please refer to CA 9.3.2).

Further, the following two papers were identified during EFSA-compliant literature search and were considered relevant for assessment on full-text level.

Data point addressed:	CA B.9.4.2
Reference:	KCA 8.4.2/0001
Author(s) (year):	Ohri, P., Sohal, S.K., Bhardwaj, R., Khurma, U.R. (2008)
Title:	STUDIES ON MELOIDOGYNE INCOGNITA UNDER INFLUENCE OF 24-EPIBRASSINOLIDE
Published:	Yes
Published in:	Annals of Plant Protection Sciences, 2008, 16 (1), 198-202
Test guideline used:	None
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional information**

Executive summary (Abstract) provided by the applicant:
The influence of 24-Epibrassinolide (EBI) was investigated on the development of root-knot nematode, *Meloidogyne incognita*. Two types of treatments were given to the nematode - direct and indirect. In direct treatment, the egg masses of *M. incognita* were exposed to six concentrations (ranging from 10^{-10} to 10^{-5} M) of EBI for 7 days. Observations recorded revealed higher percentage of hatching in treated egg masses as compared to control. Hatched juveniles from treated egg masses were further allowed to develop on plants for 45 days. EBI treated juveniles resulted into more gall numbers and larger sizes of galls as compared to untreated ones. Indirect treatment involved the treatment of different plant parts *viz.* seeds, roots and leaves of tomato cv. Pusa Ruby with six concentrations of EBI and, then untreated J_2 @ $10J_2$/g soil were released on these treated plants. Seed and root-dip treatments of tomato plants with EBI resulted in higher number of galls

[35] KCA 6.2.1/0002: Nishikawa, N. Toyama, S. Shida, A. Futatsuya, F. (1994): THE UPTAKE AND THE TRANSPORT OF 14C-LABELED EPIBRASSINOLIDE IN INTACT SEEDLINGS OF CUCUMBER AND WHEAT Report No.: na (092-088) Journal of Plant Research, 1994, 107, 125-130 Not GLP, published

and greater egg mass number as compared to control plants whereas foliar spray had not much effect on nematode development

Discussion by the applicant:

Treatment of 24-Epibrassinolide had an overall positive effect on the development of *Meloidogyne incognita.* Direct treatment of eggs and indirect treatment via seed and root-dip treatments of tomato plants both lead to greater gall numbers and in vitro treatment of eggs confirmed previous findings that 24-Epibrassinolide enhances nematode growth. However, no significant differences in growth parameters of nematodes were observed when 24-Epibrassinolide was applied by foliar spray to plants. Lesser numbers of galls and egg masses were reported for lower concentrations, and no difference to the control was found for higher concentrations. Like all plant feeding insects, root-knot nematodes are naturally exposed to brassinolide through intake of plant sap. It is therefore expected that the reported effects on root-knot nematodes occur also under natural conditions. Further, brassinolide-related steroids were reported to have been extracted from galls of root-knot nematodes (**Arima *et al.*, 1984**[36]) and **Kyndt *et al.*, 2012**[37] reported that the activation of the brassinosteroid pathway might be important for nematodes to overcome root defence. As 24-Epibrassinolide is intended to be used via foliar spray application, it can be expected that no significant effects on *Meloidogyne incognita* will occur when 24-Epibrassinolide is used according to GAP.

KCA 8.4.2/0001	**Comment RMS:** The referenced paper focusses on the effects of 24-Epibrassinolide to the nematode and plant parasite *Meloidogyne incognita.* The used experimental method is described properly. If applied by foliar spray on tomato plants (in concentrations of 10^{-10} – 10^{-5} mol/L) 24-Epibrassinolide did not show significant differences compared to the control regarding nematode growth parameters like gall size and number. This public literature study is considered as additional information by RMS, providing evidence that exposure to 24-Epibrassinolide is considered unlikely to have adverse effects to soil nematodes. It is noted that nematodes are not among the standard test organisms for soil according to the data requirements, therefore this study is of limited relevance.

Data point addressed:	CA B.9.4.2
Reference:	KCA 8.4.2/0002
Author(s) (year):	Kaur, R., Ohri, P., Bhardwaj, R. (2013)
Title:	ALTERATIONS IN ANTIOXIDATIVE ENZYMES IN MELOIDOGYNE INCOGNITA FEMALES TREATED WITH 24-EPIBRASSINOLIDE
Published:	Yes
Published in:	Indian Journal of Nematology, 2013, 43 (2), 219-221
Test guideline used:	None
Deviations:	Not applicable
GLP:	No
Acceptability:	**No**

Executive summary provided by the applicant:

The role of 24-Epibrassinolide in altering the specific activity of various antioxidative enzymes in female root-knot nematodes, *Meloidogyne incognita,* was assessed. *M. incognita* females were treated with different concentrations (10^{-11}, 10^{-9} and 10^{-7} M) of 24-Epibrassinolide. After extraction, the supernatant was used to estimate the enzyme activity of catalase, superoxide dismutase (SOD), glutathione-S-transferase (GST), esterases and protein content. Catalase activity was found to be suppressed significantly at higher concentrations; SOD activity significantly increased at lower concentrations and no significant alteration in GST activity was reported. Esterases showed a significant decrease with increase in concentration. It

[36] KCA 8.4.2/0003: Arima, M., Yokota, T., Takahashi, N. (1984): IDENTIFICATION AND QUANTIFICATION OF BRASSINOLIDE-RELATED STEROIDS IN THE INSECT GALL AND HEALTHY TISSUES OF THE CHESTNUT PLANT, Report No.: na (092-008) Phytochemistry, 1984, 23 (8), 1587-1591, Not GLP, published

[37] KCA 8.4.2/0004: Kyndt, T., Denil, S., Haegeman, A., Trooskens, G., Bauters, L., Van Criekinge, W., De Meyer, T., Gheysen, G. (2012): TRANSCRIPTIONAL REPROGRAMMING BY ROOT KNOT AND MIGRATORY NEMATODE INFECTION IN RICE, Report No.: na (092-108), New Phytologist, 2012, 196, 887-900; doi: 10.1111/j.1469-8137.2012.04311.x, Not GLP, published

was concluded that 24-Epibrassinolide might play a direct or indirect role in general metabolic processes of nematode enzymes. **Discussion by the applicant:** The methodology of sample preparation is not described in detail, e.g. it is not mentioned how the nematodes were treated with 24-Epibrassinolide and it is unclear how long after treatment the measurements were carried out. Further, the results only indicate the influence of 24-Epibrassinolide on certain hormones and it remains unclear, which effect this would have had on the nematodes in vivo. Therefore, the publication is not considered to provide relevant information for this dossier and not considered further.	
KCA 8.4.2/0002	**Comment RMS:** Agree with the discussion by the applicant. This public literature study does not provide relevant or reliable information to be used within the active substance evaluation of 24-Epibrassinolide.

Overall RMS conclusion – Effects on non-target soil meso- and macrofauna (other than earthworms):
No laboratory studies were submitted by the notifier to address the effects on non-target soil organisms (other than earthworms). No explicit data was presented by the applicant regarding natural background concentrations of 24-Epibrassinolide in soil. However, in the Fate section (Vol. 3 CA B.8.1.1.1) it is referenced, that Heumann *et al*. (2011)[38] studied the phytosterol content in soil samples. The samples were taken from different soil types such as podzoles, gleysoles cambisols and intermediates. In the measured soil samples the overall sterol concentrations ranged between 100 and 3600 mg/kg soil, the concentrations of the brassinosteroid precursor β-sitosterol ranged from 1 – 100 mg/kg soil. Due to the relative structural similarity the referenced sterols are considered suitable to serve as a proxy to estimate the order of magnitude of 24-Epibrassinolide concentration in soil.
Worst case PECsoil values were calculated in the fate section (please refer to Vol. 3 CP B 8). Following the maximum application rate of 3 x 0.05 g a.s./ha in grapes the resulting PECsoil is 0.0002 mg a.s./kg soil (with worst case assuptions of 0 % crop interception and a $DT_{50\ soil}$ of 69.02 days) and covers the GAP of all other uses. This maximum worst case PECsoil of 0.0002 mg a.s./kg soil is around ~ 5000 times below the lowest reported soil sterol concentrations (i.e. 1 mg β-sitosterol/kg soil). This supports that the natural exposure to brassinosteroids (including 24-Epibrassinolide) can be considered to be higher than the exposure following an application of the active substance in the form of a plant protection product. A low acute toxicity was demonstrated in the standard non-target arthropods species *A. rhopalosiphi* and *T. pyri* (studies with the formulated product "Sunergist").
Moreover free phytohormones such as 24-Epibrassinolide in soil are considered to be readily taken up by plants via roots.[39] Thus, it is to be expected that in case brassinosteroids occur freely in soil, e.g. if brassinosteroids are released by degradation of organic plant matter or during the use of brassinosteroid-containing plant protection products, brassinosteroids are taken up by roots and subsequently metabolised by plants.

In conclusion the waiving of the effect studies with non-target soil organisms (other than earthworms) is considered acceptable. Adverse effects posed by 24-Epibrassinolide are considered unlikely and the data requirement is considered sufficiently addressed.

B.9.5. EFFECTS ON SOIL NITROGEN TRANSFORMATION

A waiver is requested by the applicant for the performance of the standard effect studies on soil microbial activity because:

[38] KCA 7.1.1.1/0005: Heumann, S., Schlichting, A., Böttcher, J., Leinweber, P. (2011): Sterols in soil organic matter in relation to nitrogen mineralization in sandy arable soils, J. Plant Nutr. Soil Sci., 2011, 174, 576-586; doi: 10.1002/jpln.200900273, Not GLP, published

[39] KCA 6.2.1/0002: Nishikawa, N. Toyama, S. Shida, A. Futatsuya, F. (1994): THE UPTAKE AND THE TRANSPORT OF 14C-LABELED EPIBRASSINOLIDE IN INTACT SEEDLINGS OF CUCUMBER AND WHEAT Report No.: na (092-088) Journal of Plant Research, 1994, 107, 125-130 Not GLP, published

- 24-Epibrassinolide is ubiquitous in plants and plant material.
- Free 24-Epibrassinolide in soil will be absorbed by plants and thus not interact with soil processes such as nitrogen transformation (please refer to Vol. 3 CA Part B 8 B.8.1.4).
- Tsavkelova *et al*. (2006) reported that the fungus *Cercospora archidicola* is a brassinosteroid producer as well as the green alga *Chlorella vulgaris* (Stirk et al., 2013, reference KCA 8.2.6/0002, evaluated under point CA B.9.2.6).
- As brassinosteroids are phylogenetically ancient phytohormones, evolved in the Pre-Cambrian, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones.

Brassinosteroids including 24-Epibrassinolide are therefore naturally present in soil systems and no negative effects on soil microbial activity are expected.

Data point addressed:	CA B.9.5
Reference:	KCA 8.5/0001
Author(s) (year):	Tsavkelova, E.A., Klimova, S.Y., Cherdyntseva, T.A., Netrusov, A.I. (2006)
Title:	HORMONES AND HORMONE-LIKE SUBSTANCES OF MICROORGANISMS: A REVIEW
Published:	Yes
Published in:	Applied Biochemistry and Microbiology, 2006, 42 (3), 229-235
Test guideline used:	None
Deviations:	Not applicable
GLP:	No
Acceptability:	**No**

Abstract provided by RMS:

Data from the literature on the ability of microorganisms to form plant hormones have been reviewed. The substances covered include abscisic acid, ethylene and other compounds with phytohormone-like properties (brassinosteroids, oligosaccharines) and analogues of animal neurotransmitters (biogenic amines). Pathways whereby the substances are metabolized and their effects on the development and activity (physiological and biochemical) of the microorganisms are considered. The role of phytohormones and hormone-like substances in the formation of association (microorganism-host) interactions are analyzed. The potential utilities of microorganisms producing hormones and hormone-like substances are discussed.

KCA 8.4.2/0002	**Comment RMS:** The referenced public literature is a review paper focussing on the ability of microorganisms to produce hormones and hormone-like substances including phytohormones. It is mentioned, that the funghi *Cercospora archidicola* (causative agent of peanut cercosporosis) and the green alga *Chlorella vulgaris* are capable of producing brassinosteroids. Further it is mentioned that in industrial microbiology phytohormone-producing microorganisms and microbial phytohormone degraders are known and commercially used, like for example certain mycobacteria capable to cleave and modify steroids (including compounds with brassinosteroid substituents). In the review paper soil microorganisms are not explicitly mentioned. This public literature study does not provide explicit information regarding the effects of brassinosteroids to soil microorganisms, but supports the argument that microorganisms in general - and thus also microorganisms present in soil - are likely to be able to metabolise brassinosteroids. Although it is considered reasonable that adverse effects on the soil nitrogen transformation following an exposure to 24-Epibrassinolide are unlikely, the presented study is not considered sufficiently reliable or relevant for a definitive conclusion.

Comment RMS: No studies were submitted by the notifier to address the effects on soil nitrogen transformation. No explicit data was presented by the applicant regarding natural background concentrations of 24-Epibrassinolide in soil. The public literature study by Tsavkelova *et al.* (2006) does not provide explicit information regarding the effects on soil nitrogen transformation of brassinosteroids to soil microorganisms, but supports the argument that microorganisms are likely to be able to metabolise brassinosteroids present in soil (e.g. that common soil organisms like alage or funghi are capable of producing and cleaving steroid hormones). Although it is considered reasonable that adverse effects on the soil nitrogen transformation following an exposure to 24-Epibrassinolide are unlikely, the presented study is not considered sufficiently reliable or relevant for a definitive conclusion.
However in the Fate section (Vol. 3 CA B.8.1.1.1) it is referenced, that Heumann *et al.* (2011)[40] studied the phytosterol content in soil samples. The samples were taken from different soil types such as podzoles, gleysoles cambisols and intermediates. In the measured soil samples the overall sterol concentrations ranged between 100 and 3600 mg/kg soil, the concentrations of the brassinosteroid precursor β-sitosterol ranged from 1 – 100 mg/kg soil. Due to the relative structural similarity the referenced sterols are considered suitable to serve as a proxy to estimate the order of magnitude of 24-Epibrassinolide concentration in soil.
Worst case PECsoil values were calculated in the fate section (please refer to Vol. 3 CP B 8). Following the maximum application rate of 3 x 0.05 g a.s./ha in grapes the resulting PECsoil is 0.0002 mg a.s./kg soil (with worst case assuptions of 0 % crop interception and a $DT_{50\ soil}$ of 69.02 days) and covers the GAP of all other uses. This maximum worst case PECsoil of 0.0002 mg a.s./kg soil is around ~ 5000 times below the lowest reported soil sterol concentrations (i.e. 1 mg β-sitosterol/kg soil). This supports that the natural exposure to brassinosteroids (including 24-Epibrassinolide) can be considered to be higher than the exposure following an application of the active substance in the form of a plant protection product.
Moreover free phytohormones such as 24-Epibrassinolide in soil are considered to be readily taken up by plants via roots.[41] Thus, it is to be expected that in case brassinosteroids occur freely in soil, e.g. if brassinosteroids are released by degradation of organic plant matter or during the use of brassinosteroid-containing plant protection products, brassinosteroids are taken up by roots and subsequently metabolised by plants.

In conclusion the waiving of the effect studies on soil nitrogen transformation is considered acceptable. Adverse effects posed by 24-Epibrassinolide are considered unlikely and the data requirement is considered sufficiently addressed.

B.9.6. EFFECTS ON TERRESTRIAL NON-TARGET HIGHER PLANTS

A waiver is requested by the applicant for the performance of the standard effect studies on non-target plants because brassinosteroids and 24-Epibrassinolide are naturally synthesised by higher and lower plant species and therefore ubiquitous in the plant kingdom. Brassinosteroids, including 24-Epibrassinolide are naturally occurring, plant growth promoting molecules, present in higher plants, lower plants, including algae, mosses, the "living fossil" *Equisetum* as well as some fungi. Brassinosteroids are present in various plant organs such as pollen, anthers, seeds, leaves, stems, roots, flowers, grains and fruits with the highest concentrations found in pollen, seeds and fruits and thus considered an obligatory plant constituent (Bajguz, 2011). Further Bajguz (2011) reported, that 69 brassinosteroids have been isolated from 64 plant species including 53 angiosperms (12 monocotyledons and 41 dicotyledons), 6 gymnosperms, 1 pteridophyte *(Equisetum arvense),* 1 bryophyte *(Marchantia polymorpha)* and 3 algae *(Chlorella vulgaris, Cystoseira myrica* and *Hydro-dictyon reticulatum).*

[40] KCA 7.1.1.1/0005: Heumann, S., Schlichting, A., Böttcher, J., Leinweber, P. (2011): Sterols in soil organic matter in relation to nitrogen mineralization in sandy arable soils, J. Plant Nutr. Soil Sci., 2011, 174, 576-586; doi: 10.1002/jpln.200900273, Not GLP, published

[41] KCA 6.2.1/0002: Nishikawa, N. Toyama, S. Shida, A. Futatsuya, F. (1994): THE UPTAKE AND THE TRANSPORT OF 14C-LABELED EPIBRASSINOLIDE IN INTACT SEEDLINGS OF CUCUMBER AND WHEAT Report No.: na (092-088) Journal of Plant Research, 1994, 107, 125-130 Not GLP, published

24-Epibrassinolide elicits and activates the plant's self-defence mechanisms mediating the plant's resistance to unfavourable environmental conditions, (e.g. salinity, drought, cold and heat stress) and fungal diseases (Kang & Guo, 2011). Positive effects on crops are described by Khripach *et al.* (2000), like for example that the field application of 24-Epibrassinolide to barley plants in doses of 5-15 mg a.s./ha significantly decreased the extent of fungal induced leaf diseases, while the crop yield increased. Houimli *et al.* (2010) described an increased tolerance to NaCL-stress of pepper plants (*Capsicum annuum* cv.) after treatment with 24-Epibrassinolide. Bajguz & Hayat (2009) referenced, that brassinosteroids are required for normal plant development and the application of brassinosteroids leads to a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, induction of protein synthesis and the production of various chemical defence compounds.

Therefore, no adverse effect of 24-Epibrassinolide on non-target plants is expected and no studies are considered necessary for non-target terrestrial plants.

Data point addressed:	CA B.9.6
Reference:	KCA 8/0012
Author(s) (year):	Bajguz, A. (2011)
Title:	BRASSINOSTEROIDS – OCCURRENCE AND CHEMICAL STRUCTURES IN PLANTS
Published:	Yes
Published in:	Hayat, S., Ahmad, A.: BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE. Springer Verlag, 2011, Chapter 1, 1-27, DOI 10.1007/978-94-007-0189-2_1; ISBN: 978-94-007-0188-5.
Test guideline used:	No
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional information**

Abstract provided by RMS:

Brassinosteroids (BRs) are a class of plant polyhydroxysteroids that have been recognized as a new kind of phytohormones that play an essential role in plant development. BRs occur at low concentrations throughout the plant kingdom. They have been detected in all plant organs (pollen, anthers, seeds, leaves, stems, roots, flowers, and grains) and also in the insect and crown galls. BRs are structurally related to animal and insect steroid hormones. Natural 69 BRs identified so far, have a common 5a-cholestan skeleton, and their structural variations come from the kind and orientation of oxygenated functions in rings A and B. As regards the B-ring oxidation, BRs are divided into 7-oxalactone, 6-ketone (6-oxo) and 6-deoxo (non-oxidized). These steroids can be classified as C_{27}, C_{28} or C_{29} BRs depending on the alkyl-substitution on the C-24 in the side chain. In addition to free BRs, sugar and fatty acid conjugates have been also identified in plants.

KCA 8/0012	**Comment RMS:** This public literature is a chapter in a scientific book about brassinosteroids. It is referenced that brassinosteroids, including 24-Epibrassinolide are naturally synthesised by higher and lower plant species (in numerous monocotyle and dicotyle angiosperms and gymnosperms) and are present in various plant organs such as pollen, anthers, seeds, leaves, stems, roots, flowers, grains and fruits. Therefore this public literature is used as additional information supporting the argument that brassinosteroids occur naturally in plants and thus adverse effects posed by 24-Epibrassinolide are considered unlikely.

Data point addressed:	CA B.9.6
Reference:	KCA 8/0012
Author(s) (year):	Kang, Y., Guo., S. (2011):
Title:	ROLE OF BRASSINOSEROIDS ON HORTICULTURAL CROPS.
Published:	Yes

Published in:	Hayat, S., Ahmad, A. (eds.): BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE. Springer Verlag, 2011, Chapter 9, 269-288, DOI 10.1007/978-94-007-0189-2_9; ISBN: 978-94-007-0188-5.
Test guideline used:	No
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional information**

Abstract provided by RMS:

With the progress of chemical synthesis technology, structurally modified brassinosteroids (BRs) with greater stability, under field conditions have been synthesized on a commercial scale and registered as plant growth regulators for specific horticultural crops. In both fundamental and application-oriented research, BRs and their analogues play prominent roles in various physiological processes including, seed development and germination, flower sex expression, fruit development, improvement of quantity and quality of crops, and resistance to various biotic and abiotic stresses. It is worthy to note here that the involvement of BRs in plant protection from adverse environmental stress and pesticides seems to have good prospects, since BRs appear nontoxic and environmentally friendly. It is well known that horticultural crops have a great variety of produce organs as well as high yield and output values. Moreover, their production is susceptible to sub-optimum environmental conditions, especially in facilities cultivation. Thus, practical application of BRs to horticultural crops for enhancing crops production and protection may have a promising prospect in the near future.

KCA 8/0012	**Comment RMS:** This public literature is a chapter in a scientific book about brassinosteroids. It is referenced that brassinosteroids, including 24-Epibrassinolide correlate with increased tolerance of plants against certain types of environmental stress, e.g. heavy metal stress, thermal stress or pathogen stress. Therefore this public literature is used as additional information supporting the argument that brassinosteroids occur naturally in plants and thus adverse effects posed by 24-Epibrassinolide are considered unlikely.

Data point addressed:	CA B.9.6
Reference:	KCA 8.1.2.1/0002
Author(s) (year):	Khripach, V., Zhabinskii, V., De Groot, A. (2000)
Title:	TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY
Published:	Yes
Published through:	Annals of Botany, 2000, 86, 441-447; doi:10.1006/anbo.2000.1227
Test guideline used:	Not specified
Deviations:	Not specified
GLP:	No
Acceptability:	**Yes - Additional information**

Abstract provided by RMS:

The discovery of brassinosteroids (BS) just over 20 years ago opened a new era in studies of bio-regulation in living organisms. Previously, the only known role of steroids as hormones was in animals and fungi; now a steroidal hormone in plants had been added. Progress in brassinosteroid research has been very rapid. Only 20 years passed between the discovery of brassinolide, the first member of the series, and the application of brassinosteroids in agriculture. Although the other plant hormones have been studied for a much longer period, there has not been similar development. Within the last couple of years two books on brassinosteroids (Khripach VA, Zhabinskii VN, de Groot A. 1999. Brassinosteroids—a new class of plant hormones. San Diego: Academic Press; Sakurai A, Yokota T, Clouse SD, eds. 1999. Brassinosteroids: steroidal plant hormones. Tokyo: Springer Verlag) have been published, but many new data have appeared since that time. Many of the more recent data is devoted to molecular biological aspects of BS and has helped to create a vision of their role in plants and their mechanisms of action. New discoveries of the physiological properties of BS allow us to consider them as highly promising, environmentally-friendly, natural substances suitable for wide application in plant protection and yield promotion in agriculture. This aspect of BS is the main subject of this Botanical Briefing.

KCA 8.1.2.1/0002	**Comment RMS:** This public literature is a review about twenty years of brassinosteroid research.

	Positive effects on plant healt and crop yield are referenced, for example that the field application of 24-Epibrassinolide to barley plants in doses of 5-15 mg a.s./ha significantly decreased the extent of fungal induced leaf diseases, while the crop yield increased. Another protective effect agains fungal infection was referenced for cucumber plants, where first seeds were treated with a 0.1 mg a.s./L 24-Epibrassinolide solution and later the emerged plants were sprayed with 25 mg a.s./ha. Therefore this public literature is used as additional information supporting the argument that adverse effects on non-target plants posed by exposure to 24-Epibrassinolide are considered unlikely.

Data point addressed:	CA B.9.6
Reference:	KCA 8.6/0002
Author(s) (year):	Houimli, S.I.M., Denden, M., Mouhandes, B.D. (2010)
Title:	EFFECTS OF 24-EPIBRASSINOLIDE ON GROWTH, CHLOROPHYLL, ELECTROLYTE LEAKAGE AND PROLINE BY PEPPER PLANTS UNDER NaCl-STRESS
Published:	Yes
Published in:	EurAsian Journal of BioSciences, 2010, 4, 96-104; DOI: 10.5053/ejobios.2010.4.0.12
Test guideline used:	No
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional information**

Abstract provided by RMS:

Brassinosteroids are steroidal phytohormones that have the ability to overcome plant environmental stress. This study was carried out to investigate the role of 24-Epibrassinolide in inducing pepper plant salt tolerance as measured by a range of physiological parameters: growth, chlorophyll, electrolyte leakage and proline. Capsicum annuum cv. Beldi seedlings were sprayed with 24-Epibrassinolide both in the presence or the absence of NaCl and were sampled, 28 days after treatments. As a result of analysing the cultures under salinity stress, it was determined that the biomass and the chlorophyll decreased significantly, while the electrolyte leakage and the proline concentration increased considerably under salinity stress. However, the application of 24-Epibrassinolide significantly ameliorated the adverse effects of salinity on the examined parameters, confirming the suppositions of previous authors who have claimed that exogenously applied 24-Epibrassinolide can increase growth and protect the integrity of the cellular membrane in stressed plants.

KCA 8.6/0002	**Comment RMS:** This public literature is a study about the effects of 24-Epibrassinolide on induced salinity stress in pepper plants. The results showed that NaCl-stress (irrigation with salt water) caused an overall growth reduction of plants (reduced masses of fresh and dry leaf material). The treatment of NaCl-stressed plants with a foliar application of 0.5 mg/L 24-Epibrassinolide solution reduced the adverse effects of fresh and dry leaf weight reduction. Although no information is given regarding the application rate (in mg a.s./ha) is provided this public literature is considered useful as supplemental information supporting the argument that adverse effects on non-target plants posed by exposure to 24-Epibrassinolide are considered unlikely.

Data point addressed:	CA B.9.6
Reference:	KCA 8/0091
Author(s) (year):	Bajguz, A., Hayat, S. (2009)
Title:	EFFECTS OF BRASSINOSTEROIDS ON THE PLANT RESPONSES TO ENVIRONMENTAL STRESSES
Published:	Yes
Published in:	Plant Physiology and Biochemistry, 2009, 47, 1-8; doi:10.1016/j.plaphy.2008.10.002.

Test guideline used:	No
Deviations:	Not applicable
GLP:	No
Acceptability:	**Yes - Additional information**

Abstract provided by RMS:

Brassinosteroids are found in a wide range of organisms from lower to higher plants. They are steroidal plant hormones implicated in the promotion of plant growth and development. Brassinosteroid metabolism has long been known to be altered in plants responding to abiotic stresses and to undergo profound changes in plants interacting with bacterial, fungal and viral pathogens. This review describes the role of brassinosteroids in response to various kinds of stresses via activation of different mechanisms.

KCA 8/0091	**Comment RMS:** This public literature is a review about the effects of brassinosteroids on plant responses to environmental stress. It is referenced that plants require brassinosteroids for normal development and growth. Furter is is described how brassinosteroids increase tolerance to abiotic and biotic stresses in plants on a molecular level. Therefore this public literature is considered useful as additional information supporting the argument that adverse effects on non-target plants posed by exposure to 24-Epibrassinolide are considered unlikely.

Overall RMS conclusion – Effects on terrestrial non-target higher plants:

No effect studies were submitted by the notifier to address the effects on terrestrial non-target plants, but the public literature studies above support that brassinosteroids are commonly found in plant tissue and no adverse effects are reported. In contrast it was reported that treatment of crop plants with brassinosteroids, including 24-Epibrassinolide increased crop health and yield.

Furthermore in efficacy trials with the product Sunergist no effects on phytotoxicity and vegetative vigour were found in grapes (with application rates up to 0.8 L product/ha in 600-800 L water), in lettuce (with application rates up to 0.8 L product/ha in 400 L water) and in sugar beet (with application rates up to 0.2 L product/ha in 200 L water). For further details please refer to Vol. 3 CP B3.

Therefore RMS concludes that waiving the effect studies on terrestrial non-target plants can be considered acceptable, adverse effects on non-target plants induced by 24-Epibrassinolide are considered unlikely and the data requirement as sufficiently addressed.

B.9.6.1. Summary of screening data

Please refer to B.9.6.

B.9.6.2. Testing on non-target plants

Please refer to B.9.6.

B.9.7. EFFECTS ON OTHER TERRESTRIAL ORGANISMS (FLORA AND FAUNA)

The following paper was identified during EFSA-compliant literature search and was considered relevant for assessment on full-text level.

Data point addressed:	CA B.9.7
Reference:	KCA 8.7/0001
Author(s) (year):	Waisi, H., Marko, M., Nedeljkovic, N.S., Ormai, M., Nikolic, B., Tatjana, P., Raicevic, V. (2015)
Title:	BACTERIOSTATIC EFFECT OF 24-EPIBRASSINOLIDE AGAINST ERWINIA AMYLOVORA ISOLATES
Published:	Yes
Published in:	Acta Microbiologica Hellenica, 2015, 60 (3), P 14B, 189
Test guideline used:	None

Deviations:	Not applicable
GLP:	No
Acceptability:	**No**

Executive summary provided by the applicant:

24-Epibrassinolide was found to have a concentration dependent inhibitory effect on *Erwinia amylovora* under laboratory conditions. Four isolates of *Erwinia amylovora* were tested. Concentration of $5.2*10^{-6}$ M had an inhibitory effect on 2 isolates, $5.2*10^{-11}$ M had an inhibitory effect on 4 isolates. All other concentrations (from $5.2*10^{-5}$ to 10^{-15} M) had no effect.

Discussion by the applicant:

The available full-text is a short summary of a poster presentation, which did not include enough information to evaluate the found effects thoroughly. For example, it is not clear to which extend inhibitory effects were observed in the affected isolates and the full methodology of the test is not presented (e.g. how many replicas, statistical significance of the test).
In addition, this effect was only observed for two not adjacent concentrations out of the approximately 11 concentrations tested. It is further unclear, if this in vitro effect would also occur outside laboratory conditions.
Therefore, the reported effect is not considered relevant for the use of 24-Epibrassinolide according to GAP.

KCA 8.4.2/0002	**Comment RMS:** Agree with the discussion by the applicant. This public literature study does not provide relevant or reliable information to be used within the active substance evaluation of 24-Epibrassinolide.

Overall RMS conclusion – Effects on other terrestrial organisms (flora and fauna):

No other data concerning effects of the active substance to other terrestrial non-target organisms are available. As such data are not a mandatory requirement, the data requirement is considered sufficiently addressed.

B.9.8. EFFECTS ON BIOLOGICAL METHODS FOR SEWAGE TREATMENT

A waiver is requested by the applicant for the performance of the standard effect studies on effects on biological methods for sewage treatment because:

- 24-Epibrassinolide is only soluble in low quantities in water and will be absorbed by plants when available.
- 24-Epibrassinolide is ubiquitous in plant and plant material and is therefore a natural component in sewage sludge

As a consequence, adverse effects on biological methods for sewage treatment from the application of 24-Epibrassinolide are considered unlikely.

Comment RMS: No effect studies were submitted by the notifier to address the effects on biological methods for sewage treatment. No data was presented by the applicant regarding natural background concentrations of 24-Epibrassinolide in sewage or sewage sludge. However taking into account that brassinosteroids naturally occur in plant matter and the environment the waiver is considered acceptable. Further it is reported in the public literature study of Tsavkelova *et al.* (2006, please refer to KCA 8.5/0001) that microorganisms in general are capable to metabolise 24-Epibrassinolide. This provides (together with the low predicted environmental concentrations) evidence that adverse effects to sewage sludge induced by 24-Epibrassinolide are considered unlikely and the data requirement was sufficiently addressed.

B.9.9. MONITORING DATA

Monitoring data concerning effects of the active substance to non-target organisms are not available and are not a mandatory requirement.

B.9.10. REFERENCES RELIED ON

Literature search

A literature search (KCA 9/0001; SCC Project No. PP309-00002, February 2017) for the active substance 24-Epibrassinolide was performed in accordance to the provisions of the EFSA Guidance "Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) 1107/2009".

The report (KCA 9/0001) summarises the search and selection process of the literature search performed.

Data point addressed:	CA B.9.10
Reference:	KCA 9/0001
Author(s) (year):	Reisinger, T., Huber, L. (2017)
Title:	LITERATURE REVIEW REPORT - ACTIVE SUBSTANCE: 24-Epibrassinolide
Laboratory report / project Number (Doc No.):	PP309-00002
Testing facility:	Not applicable
Published:	No
Test guideline used:	EFSA Guidance "Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009; EFSA Journal 2011, 9(2):2092"
Deviations:	No
GLP:	Not applicable
Acceptability:	**Yes**

The objective of the literature search was the assessment of scientific peer-reviewed open literature published within the last 10 years and dealing with side-effects on health, the environment and non-target species for the active substance 24-Epibrassinolide.

Literature was searched accessing the databases: AGRICOLA, BIOSIS, CABA, EMBASE, ESBIOBASE, HCAPLUS, MEDLINE, PASCAL, PQSCITECH, TOXCENTER via the service provider STN-International. The search strategy was based on a single concept search.

The search has been carried out on 09.11.2016 (calendar week 45).

In a first step the CAS REGISTRY database was accessed and the CAS number was searched to retain information on identity and substance names/synonyms.
The following list of substance specific search terms generated was used as query to search the STN databases:

CAS Number: 78821-43-9

Chemical names:

- (1R,3AS,3BS,6AS,8S,9R,10AR,10BS,12AS)-1-((1S,2R,3R,4R)-2,3-DIHYDROXY-1,4,5-TRIMETHYLHEXYL)HEXADECAHYDRO-8,9-DIHYDROXY-10 A,12A-DIMETHYL-6H-BENZ(C)INDENO(5,4-E)OXEPIN-6-ONE
- (22R,23R,24R)-2.ALPHA.,3.ALPHA.,22,23-TETRAHYDROXY-B-HOMO-7-OXA-5.ALPHA.-ERGOSTAN-6-ONE
- EPIBRASSINOLIDE R
- EPIBRASSINOLIDE
- 126721-49-1
- 24(R)-EPIBRASSINOLIDE
- 24-EPI-BRASSINOLIDE
- 24-EPIBRASSINOLIDE

- 6H-BENZ(C)INDENO(5,4-E)OXEPIN-6-ONE, 1-(2,3-DIHYDROXY-1,4,5 TRIMETHYLHEXYL)HEXADECAHYDRO-8,9-DIHYDROXY-10A,12ADIMETHYL-,(1R-(1.ALPHA.(1S*,2R*,3R*,4R*),3A.BETA.,3B.ALPHA.,6A.BETA.,8.BETA.,9.BETA.,10A/BI
- 72075-02-6
- 78821-43-9

Trade name:

- EPIN

In total, 3861 hits were retrieved, which were further filtered by publication type (patents were excluded) and date (publication year >2005).
After removal of duplicates, 854 records were retrieved form bibliographic databases and screened for relevance by expert reviewers based on their titles.

At this step, 802 references were excluded.

For the remaining 52 records, complete reference information and abstracts were retrieved from the bibliographic databases and screened by the reviewers for relevance with respect to the relevant EUdata requirements related to side-effects on human health, non-target species and the environment for the active substance 24-Epibrassinolide.

At this step, 43 references were excluded.

Table 9.10-1: Overview on records retireved and study selection process

Results of the study selection process	**n**
Total number of summary records retrieved after all searches of peer-reviewed	854
Number of summary records excluded from the search results after assessment screening of the summary records (title / abstract) for relevance	845
Number of summary records retrieved from bibliographic databases	54
Total number of full-text documents assessed in detail	9
Number of full text documents included in the assessment	8

Nine full-text documents were assessed in detail based on the Klimisch score (J. Klimisch, M. Andreae and U. Tillmann (1997) A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data Regulatory Toxicology and Pharmacology Vol 25 pp 1-5).

The list of relevance criteria (modified Klimisch score) is displayed in the following table.

Table 9.10-2: Modified Klimsch criteria for relevance for each data requirement

Data requirements (indicated by the corresponding EU data point)	**Criteria for relevance**	
General criteria for relevance considered for all data requirements indicated by the corresponding EU data points	1	Publication scientifically sound
	2	Does provide relevant information for dossier preparation or risk assessment purpose
	3	Method validated
	4	Documentation sufficient for assessment
	5	Does meet important criteria of today standard methods
	6	No relevant methodological deficiencies
	7	Suitable test system
	8	Document does not contain already identified results

As the relevance of the publications was evaluated by the (modified) Klimisch score, the score for the excluded publications uses the negation of the above listed criteria (e.g. Criterium 3“Method validated” would turn to “Method NOT validated” if the method is not validated and the publication is excluded based on this criterium.

Nine full-text documents were assessed in detail. One of these publications did not provide relevant information for the dossier preparation or risk assessment purposes and was as well assessed as obviously not relevant for the EU data requirements (please refer to Table 9.10-4).

After excluding one full text, eight publications were considered relevant and relieable and included in the assessment report under the respective subheadings. Five publications were selected to provide ecotox relevant information and will be cited in the supplementary dossiers under point B.9.2.6 (CA 8.2.6), B.9.3.1 (CA 8.3.1), B9.4.2 (CA 8.4.2) and B.9.7 (CA 8.7). For further information see Table 9.10-3.

9.6.2-3: Bibliographic references to all relevant studies and studies whose relevance remains unclear after detailed assessment for relevance of full-text documents, ordered by author names. Studies relevent for the ecotoxicology section are in bold.

Author(s)	Data requirement (indicated by the corresponding EU Annex point)	Year	Title	Source
Chuda-Mickiewicz, B., et al.	**CA 8.3.1**	**2009**	**The Role of Phytohormones in Instrumental Insemination of Queen Bees**	**Journal of Apicultural Science, 53(2): 91-96**
Mekhalfi, M., et al.	**CA 8.2.6**	**2012**	**Consequences of the presence of 24 - epibrassinolide , on cultures of a diatom, Asterionella formosa**	**Biochimie 94(5): 1213-1220**
Nadzharyan, L. A., et al.	CA 5.8.2	2006	Hemopoiesis- and steroid metabolism indices in animals under epibrassinolide effect	Sovremennye Problemy Toksikologii 2: 43-48
Ohri, P., et al.	**CA 8.4.2**	**2008**	**Studies on Meloidogyne incognita under influence of 24 - epibrassinolide**	**Annals of Plant Protection Sciences 16(1): 198-202**
Rarova, L., et al.	CA 5.8.2	2012	Brassinosteroids inhibit in vitro angiogenesis in human endothelial cells	Steroids, 77(13): 1502-1509
Kaur, R., et al.	**CA 8.4.2**	**2013**	**Alterations in antioxidative enzymes in Meloidogyne incognita females treated with 24 - epibrassinolide**	**Indian Journal of Nematology 43(2): 219-221**
Voitovich, A. M., et al.	CA 5.8.2 CA 5.8.4	2006	Epibrassinolide effect on immune system parameters	Sovremennye Problemy Toksikologii 3: 33-37
Waisi, H., et al.	**CA 8.7**	**2015**	**Bacteriostatic effect of 24 - epibrassinolide against erwinia amylovora isolates**	**Acta Microbiologica Hellenica, 60(3): 2015-2024**

9.6.2-4: References excluded from the assessment report after full text relevance assessment

Author(s)	Year	Title	Source	Klimsch score	Reason(s) for not including the study
Sysa, A. G., et al.,	2010	Effect of the structure of the brassinosteroid side chain on monooxygenase activity of	Prikladnaia biokhimiia i mikrobiologiia,	2 Does not provide relevant	Study shows the dependence of the side chain of

		liver microsomes	Vol. 1, pp 29-34	information for dossier preparation or risk assessment purpose	Brassinosteroids for its effect on the microsomal enzymatic system. 24-Epibrassinolde did not reveal an adverse effect up to a concentration of 250 uM. No other effect on the monooxygenase system could be determined. Thus, for 24-BR no adverse effect is shown.

Detailed lists of all studies screened can retrieved from the literature review report.

KCA 9/0001	**Comment RMS:** The literature search is compliant with the EFSA GD on open literature search (EFSA Journal 2011;9(2):2092) and is considered acceptable by RMS. The five relevant full-text documents were addressed in the respective sections.

List of data and references submitted by the applicant and relied on:

Data point	Author(s)	Year	Title Doc. No., (prev. used Doc. No.), (Report No.) Source (where different from company) GLP or GEP status, Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N
CA B.9.1.1	Nasonov, I.V. Likhacheva, M.I. Litvinovskaya, R. Sauchuk, A.L.	2016	IMMUNOSTIMULATORY PROPERTIES OF BRAVIDEFEN, A BRASSINOSTEROID-BASED DRUG, IN CHICKENS Report No.: na (092-110) 23rd Conference on Isoprenoids and National Academy of Sciences of Belarus, Chemical Series 2016, N 3, 1-128 (Posters P99-100) Not GLP, published	N	N		nr	N
CA B.9.1.2.1	Khripach, V. Zhabinskii, V. De Groot, A.	2000	TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY Report No.: na (092-029) Annals of Botany, 2000, 86, 441-447; doi:10.1006/anbo.2000.1227 Not GLP, published	N	N		nr	N

Data point	Author(s)	Year	Title Doc. No., (prev. used Doc. No.), (Report No.) Source (where different from company) GLP or GEP status, Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N
CA B.9.2.1	[redacted]	2017	ACUTE FISH TOXICITY STUDY IN FRESHWATER FISH (BRACHYDANIO RERIO) WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6122 (821-001) [redacted] GLP, unpublished	Y	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH Sunergist Co., Ltd.	N
CA B.9.2.1	Zhabinskii, V.N. Khripach, N.B. Khripach, V.A.	2015	STEROID PLANT HORMONES: EFFECTS OUTSIDE PLANT KINGDOM Report No.: na (092-099) Steroids, 2015, 97, 87-97; doi: 10.1016/j.steroids.2014.08.025 Not GLP, published	N	N		nr	N
CA B.9.2.4.1	Matlock, D. Moore, S.	2017	ACUTE TOXICITY OF 24-EPIBRASSINOLIDE TO DAPHNIA MAGNA UNDER STATIC CONDITIONS Report No.: 115SRUS16C0107 (822-001) SynTech Research Laboratory, Stilwell, Kansas, USA GLP, unpublished	N	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH Sunergist Co., Ltd.	N
CA B.9.2.5.3	Hassett, J.P. Fred Lee, G. Lee, F.G.	1977	STEROLS IN NATURAL WATER AND SEDIMENT Report No.: na (092-168) Water Research, 1977, 11, 983-989 Not GLP, published	N	N		nr	N
CA B.9.2.6	Stirk, W.A. Balint, P. Tarkowska, D. Novak, O. Strnad, M. Oerdoeg, V. van Staden, J.	2013	HORMONE PROFILES IN MICROALGAE: GIBBERELLINS AND BRASSINOSTEROIDS Report No.: na (092-051) Plant Physiology and Biochemistry, 2013, 70, 348-353; doi: 10.1016/j.plaphy.2013.05.037 Not GLP, published	N	N		nr	N
CA B.9.2.6	Mekhalfi, M. Avilan, L. Lebrun, R. Botebol, H. Gontero, B.	2012	CONSEQUENCES OF THE PRESENCE OF 24-EPIBRASSINOLIDE, ON CULTURES OF A DIATOM, ASTERIONELLA FORMOSA Report No.: na (092-109) Biochimie, 2012, 94, 1213-1220; doi: 10.1016/j.biochi.2012.02.011 Not GLP, published	N	N		nr	N

Data point	Author(s)	Year	Title Doc. No., (prev. used Doc. No.), (Report No.) Source (where different from company) GLP or GEP status, Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N
CA B.9.3.1	Chuda-Mickiewicz, B. Prabucki, J. Samborski, J. Rostecki, P.	2009	THE ROLE OF PHYTOHORMONES IN INSTRUMENTAL INSEMINATION OF QUEEN BEES Report No.: na (092-105) Journal of Apicultural Science, 2009, 53 (2), 91-96 Not GLP, published	N	N		nr	N
CA B.9.3.1.1.1	Bharathiraja, K.	2017 a	ACUTE ORAL TOXICITY STUDY IN HONEY BEES (APIS MELLIFERA) WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6125 (832-002) RCC Laboratories India Private Limited, Hyderabad, India GLP, unpublished	N	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH Sunergist Co., Ltd.	N
CA B.9.3.1.1.2	Bharathiraja, K.	2017 b	ACUTE CONTACT TOXICITY STUDY IN HONEY BEES (APIS MELLIFERA) WITH 24-EPIBRASSINOLIDE (TGAI) Report No.: 6124 (832-001) RCC Laboratories India Private Limited, Hyderabad, India GLP, unpublished	N	Y	New study necessary for the approval of 24-Epibrassinolide	Suntton GmbH Sunergist Co., Ltd.	N
CA B.9.4.2	Ohri, P. Sohal, S.K. Bhardwaj, R. Khurma, U.R.	2008	STUDIES ON MELOIDOGYNE INCOGNITA UNDER INFLUENCE OF 24-EPIBRASSINOLIDE Report No.: na (092-112) Annals of Plant Protection Sciences, 2008, 16 (1), 198-202 Not GLP, published	N	N		nr	N
CA B.9.6	Bajguz, A.	2011	BRASSINOSTEROIDS – OCCURRENCE AND CHEMICAL STRUCTURES IN PLANTS. In: Hayat, S., Ahmad, A. (editors): BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE Report No.: na (092-146) Springer Verlag, 2011, 1-477, DOI 10.1007/978-94-007-0189-2; ISBN: 978-94-007-0188-5 Not GLP, published	N	N		nr	N

Data point	Author(s)	Year	Title Doc. No., (prev. used Doc. No.), (Report No.) Source (where different from company) GLP or GEP status, Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N
CA B.9.6	Kang, Y., Guo, S.	2011	ROLE OF BRASSINOSTEROIDS ON HORTICULTURAL CROPS. In: Hayat, S., Ahmad, A. (editors): BRASSINOSTEROIDS: A CLASS OF PLANT HORMONE Report No.: na (092-146) Springer Verlag, 2011, 1-477, DOI 10.1007/978-94-007-0189-2; ISBN: 978-94-007-0188-5 Not GLP, published	N	N		nr	N
CA B.9.6	Khripach, V. Zhabinskii, V. De Groot, A.	2000	TWENTY YEARS OF BRASSINOSTEROIDS: STEROIDAL PLANT HORMONES WARRANT BETTER CROPS FOR THE XXI CENTURY Report No.: na (092-029) Annals of Botany, 2000, 86, 441-447; doi:10.1006/anbo.2000.1227 Not GLP, published	N	N		nr	N
CA B.9.6	Houimli, S.I.M. Denden, M. Mouhandes, B.D.	2010	EFFECTS OF 24-EPIBRASSINOLIDE ON GROWTH, CHLOROPHYLL, ELECTROLYTE LEAKAGE AND PROLINE BY PEPPER PLANTS UNDER NaCl-STRESS Report No.: na (092-106) EurAsian Journal of BioSciences, 2010, 4, 96-104; DOI: 10.5053/ejobios.2010.4.0.12 Not GLP, published	N	N		nr	N
CA B.9.6	Bajguz, A. Hayat, S.	2009	EFFECTS OF BRASSINOSTEROIDS ON THE PLANT RESPONSES TO ENVIRONMENTAL STRESSES Report No.: na (092-133) Plant Physiology and Biochemistry, 2009, 47, 1-8; doi:10.1016/j.plaphy.2008.10.002 Not GLP, published	N	N		nr	N

Data point	Author(s)	Year	Title Doc. No., (prev. used Doc. No.), (Report No.) Source (where different from company) GLP or GEP status, Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N
CA B.9.10	Reisinger, T. Huber, L.	2017	Literature Review Report according to EFSA Guidance "Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009; EFSA Journal 2011, 9(2):2092" Active Substance: 24-Epibrassinolide SCC Scientific Consulting Company **SCC Project No** PP309-00002 Not GLP	N			(1)Suntton Co., Ltd. ; (2)Sunergist Co., Ltd. Flat/Rm 1501(697) 15F, SPA Centre, 53-55 Lockhart Road, Wanchai, Hong Kong	N

na = not applicable / ni = not indicated / nr = not relevant

APPENDIX I

The applicant submitted an introduction to brassinosteroids in support with the dossier of 24-Epibrassinolide. This general information was not evaluated in detail by RMS because it is not deemed necessary for the evaluation. Nonetheless it is presented in this Appendix I for completeness (in *italics*) and the full references cited in the introduction can be found at the end:

Brassinosteroids, including 24-Epibrassinolide are naturally occurring, plant growth promoting molecules, present in higher plants, lower plants, including algae, mosses, the "living fossil" Equisetum as well as some fungi (Takatsuto et al., 1990a, Table A-1). Brassinosteroids are present in all plant organs such as pollen, anthers, seeds, leaves, stems, roots, flowers, grains and fruits with the highest concentrations found in pollen, seeds and fruits (Zhu et al., 2013) and considered an obligatory plant constituent. Pollen and immature seeds show contents of Brassinosteroids in a range of 0.001 – 6400 µg/kg fresh weight, while shoots and leaves usually show lower concentrations of 0.001 – 100 µg/kg fresh weight. Fruits, e,g. apples contain 10-35 µg/kg fresh weight (Table A-2). The concentration of Brassinosteroids in plants is regulated by a complex system of feedback pathways (e.g. Saini et al., 2015) and Brassinosteroids are constantly synthesised, metabolised, activated and inactivated depending on the plant's needs as well as environmental cues. The concentrations of Brassinosteroids are continuously fluctuating - spatially and temporally: in a single plant, different concentrations can be measured simultaneously in different plant organs, cell structures and cells as well as in the same location at different times (e.g. Symons et al., 2008).

Brassinosteroids represent ubiquitous, phylogenetically ancient phytohormones that promote growth in land plants as well as in green freshwater algae. According to Kutschera and Wang (2012), Brassinosteroids may have evolved in the Pre-Cambrian, at a time during the evolution of life on earth, when the split between uni- and multicellular green algae (which later gave rise to the embryophytes) had not yet occurred.

24-Epibrassinolide was first synthesized in 1979 (Thompson et al., 1979). Ten years later the natural occurrence of 24-Epibrassinolide in the plant kingdom was demonstrated by isolation and detection of 24-Epibrassinolide in Vicia faba pollen (Ikekawa et al., 1988) for the first time. Isolation of 24-Epibrassinolide and other Brassinosteroids, respectively, from natural materials is a complicated and expensive process. Therefore, 24-Epibrassinolide is chemically synthesized, identical to the naturally occurring 24-Epibrassinolide and is considered a "natural-identical synthesized molecule".

*Brassinosteroids, which belong to the class of polyhdroxysteroids, can be divided into free as well as conjugated signal molecules. They are classified by their alkyl-substitutions in the side chain, as C_{27}, C_{28} or C_{29} Brassinosteroids (**Fehler! Verweisquelle konnte nicht gefunden werden.**).*

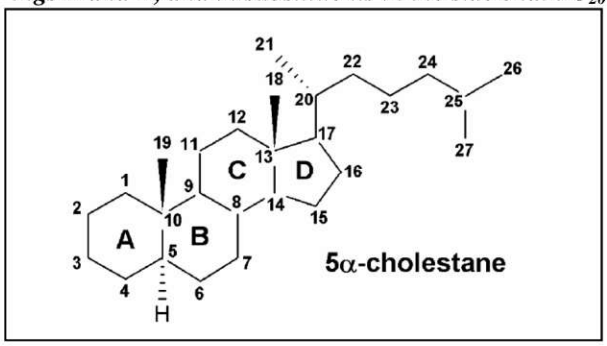
Table A-1: Division of free brassinosteroids according to number of carbon in structure and different types of B-ring and substituents in the A-ring brassinolide (BL), Castasterone (CS), Cathasterone (CT), Dolicholide (DL), Dolichosterone (DS), Dehydroteasterone (DT), Methyl (Me), Secasterone (SE), Teasterone (TE), Typhasterol (TY)

No. of carbon	*Type of B-ring*	*Substituent in A-ring*	*Representative(s)*
C27	*7-Oxalactone*	*C(2α,3α)-OH*	*28-norBL*
	6-Oxo	*C(2α,3α)-OH*	*28-norCS*
		C3α-OH	*28-norTY*
	6-Deoxo	*C(2α,3α)-OH*	*6-deoxo-28-norCS*
		C3α-OH	*6-deoxo-28-norTY, 3-epi-6-deoxo-28-norCT*
		C3β-OH	*6-deoxo-28-norTE, 6-deoxo-28-norCT*
		C3-oxo group	*3-dehydro-6-deoxo-28-norTE, 3-keto-22-epi-28-norCT*
C28	*7-Oxalactone*	*C(2α,3α)-OH*	*BL, 24-epiBL, 23-dehydroBL, DL*
		C(2α,3β)-OH	*3-epi-23-dehydroBL, 3-epiBL*
		C(2β,3α)-OH	*2-epi-23-dehydroBL*
		C(2β,3β)-OH	*2,3-diepi-23-dehydroBL*
		C3α-OH	*2-deoxyBL, 7-oxTY*
		C3β-OH	*7-oxTE*
	6-Oxo	*C(2α,3α)-OH*	*CS, 24-epiCS, DS*
		C(2α,3β)-OH	*3-epiCS, 3,24-diepiCS*
		C(2β,3α)-OH	*2-epiCS*

		C(2β,3β)-OH	*2,3-diepiCS*
		C(1β,2α,3α)-OH	*1β-OH-CS*
		C(1α,2α,3β)-OH	*3-epi-1α-OH-CS*
		C3α-OH	*TY*
		C3β-OH	*TE, CT*
		C3-oxo group	*3-DT (3-dehydroTE)*
		C(2β,3β)-epoxide	*SE, 24-epiSE*
		C(2α,3α)-epoxide	*2,3-diepiSE*
		$\Delta^{2,3}$	*Secasterol*
	6-Deoxo	*C(2α,3α)-OH*	*6-deoxoCS, 6-deoxo-24-epiCS, 6-deoxoDS*
		C(2α,3β)-OH	*3-epi-6-deoxoCS*
		C3α-OH	*6-deoxoTY, 3-epi-6-deoxoCT*
		C3β-OH	*6-deoxoTE, 6-deoxoCT*
		C3-oxo group	*6-deoxo-3DT (3-dehydro-6-deoxoTE)*
	6-Hydroxy	*C(2α,3α)-OH*	*6α-OH-CS*
C29	*7-Oxalactone*	*C(2α,3α)-OH*	*28-homoBL, 28-homoDL*
	6-Oxo	*C(2α,3α)-OH*	*28-homoCS, 28-homoDS, 25-MeDS, 25-MeCS*
		C(2β,3α)-OH	*2-epi-25-MeDS, 2-epi-25-MeCS*
		C(2β,3β)-OH	*2,3-diepi-25-MeDS, 2,3-diepi-25-MeCS*
		C3α-OH	*28-homoTY, 2-deoxy-25-MeDS*
		C3β-OH	*28-homoTE, 3-epi-2-deoxy-25-MeDS*
	6-Deoxo	*C(2α,3α)-OH*	*6-deoxo-28-homoDS, 6-deoxo-25-MeDS*

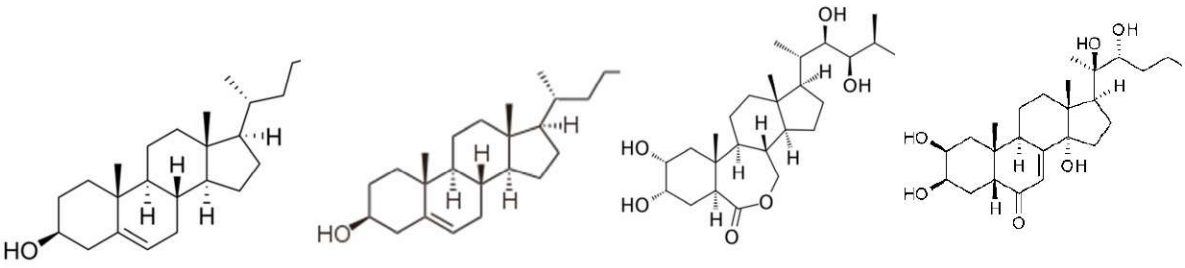
Most Brassinosteroids, including the physiologically most important C_{28} brassinolides, are synthesized by the precursor campesterol via a common 5α-cholestane skeleton. Structural variations are synthesized by differences in orientation of the oxygenated functions in rings A and B, and by different substituents in the side chain (Bajguz, 2011, Figure A-1).

Figure A-1: 5α-cholestane as the skeleton of Brassinosteroid synthesis. Structural differences occur in rings A and B, and in substituents in the side chain C_{20} – C_{27}.



Campesterol (Figure A-2a) derives from 5α-cholestane and, with its attached alcohol group, chemically represents a sterol, like e.g. the animal sterol cholesterol (Figure A-2b) or the insect derived molting hormone ecdysterone (Figure A-2d). Campesterol exhibits a double bond from carbon 5 to carbon 6 and therefore can be defined as a Δ^5 sterol. During synthesis of 24-Epibrassinolide, campesterol becomes fully saturated (Δ^0) by creation of an additional carbonyl bond and attachment of hydroxyl groups to the side chains. Therefore, 24-Epibrassinolide represents no longer a sterol but a stanol as, per definition, stanols are saturated or reduced sterols that share structural similarities with the campesterol/cholesterol skeleton. Due to the fact that alkenes (double bonds between carbons), as found in other plant steroids, display a chemical bond of higher reactivity, 24-Epibrassinolide has to be differentiated concerning its structural chemistry for the absence of an alkene group. This difference for example minimizes stanol absorption in the mammalian intestines (Bajguz, 2011).

Figure A-2: Structure of the (a) precursor Campesterol, (b) the animal derived molecule Cholesterol, (c) the active substance 24-Epibrassinolide, and (d) the insect hormone Ecdysterone.



(a) Campesterol *(b) Cholesterol* *(c) brassinolide* *(d) Ecdysteron*

24-Epibrassinolide belongs, besides 28-Homobrassinolide and Brassinolide, to the most biologically active Brassinosteroids, all three of them having similar chemical structures. 28-homobrassinolide and 24-Epibrassinolide differ from Brassinolide by the substituent in the side chain at C24 or by its configuration at C24, respectively (Khripach et al., 2000). All three act in low concentrations between 0.1 – 0.001 ppm (Ikekawa and Zhao, 1991).

Table A-2 is a summary table based on open literature and without any claim to completeness. It is to be expected that Brassinosteroids are also ubiquitous distributed in organisms not included in this table.

Table A-9.6.2-1: Natural occurrence and concentrations of Brassinosteroids in higher and lower plants, fungi, processed and unprocessed foodstuffs

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
Monocotyledons				
Arecaceae				
Date palm (Phoenix dactylifera)	*pollen*	*24-epiCS*	*unspecified*	*Zaki et al., 1993*[3]
Gramineae				
Perennial ryegrass (Lolium perenne L.)	*pollen*	*BR (1)*	*0.001*	*Taylor et al., 1993*[3]
Rice (Oryza sativa L.)	*shoot*	*BL*	*unspecified*	*Abe et al., 1984b; Abe 1991*[3]
	shoot	*CS*	*0.014*	*Abe et al., 1984b; Abe 1991*[3]
	shoot	*BR (1)*	*0.008*	*Abe et al., 1984b; Abe 1991*[3]
	bran	*BR (3)*	*unspecified*	*Abe et al., 1995a*
	seeds	*CS, BR (2)*	*unspecified*	*Park et al., 1994b*
	grains	*24-epiBL*	*216*	*Khripach et al., 2013*
	grains	*BL*	*29*	*Khripach et al., 2013*
	grains	*28-homoBL*	*4.4*	*Khripach et al., 2013*
Canary grass (Phalaris canariensis)	*seeds*	*CS*	*5*	*Shimada et al.,1996*[3]
	seeds	*BR (1)*	*0.7*	*Shimada et al.,1996*[3]
Common wheat (Triticum aestivum L.)	*grain*	*CS, BR (4)*	*unspecified*	*Yokota et al, 1994*
Rye (Secale cereal)	*seeds*	*CS, BR (4)*	*unspecified*	*Schmidt et al., 1995b*[3]
	leaves	*BR (3)*	*0.02-0.052*	*Antonchick et al., 2003*[3]
	roots	*BR (2)*	*0.032-0.107*	*Antonchick et al., 2003*[3]
Maize (Zea mays L.) - dent corn	*pollen*	*CS*	*120*	*Suzuki et al., 1986*
	pollen	*BR (2)*	*4.1-6.6*	*Suzuki et al., 1986*
	roots	*CS*	*0.3*	*Kim et al., 2000a*
Maize (Zea mays L.) - sweet corn	*pollen*	*CS*	*27.2*	*Gamoh et al., 1990*[3]
	pollen	*BR (2)*	*16.9-18.3*	*Gamoh et al., 1990*[3]
Liliaceae				
Asian fawnlily (Erythronium japonicum	*pollen, anther*	*BR (1)*	*5*	*Yasuta et al., 1995*

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
Decne)				
Lilium elegans Thunb.	*pollen*	*CS*	*10-50*	*Suzuki et al., 1994b ; Yasuta et al., 1995*
Liliaceae				
	pollen	*BL*	*1-5*	*Suzuki et al., 1994b ; Yasuta et al., 1995*
	pollen	*BR (2)*	*1-50*	*Suzuki et al., 1994b ; Yasuta et al., 1995*
Lilium longiflorum Thunb.	*pollen*	*BR (1)*	*3180*	*Abe, 1991*[3] *; Abe et al., 1994 ; Asakawa et al.,1994, 1996 ; Soeno et al., 2000*
Lilium longiflorum Thunb.	*anther*	*BL, CS*	*unspecified*	*Abe, 1991*[3] *; Abe et al., 1994 ; Asakawa et al.,1994, 1996 ; Soeno et al., 2000*
	anther	*BR (5)*	*20-2440*	*Abe, 1991*[3] *; Abe et al., 1994 ; Asakawa et al.,1994, 1996 ; Soeno et al., 2000*
Garden tulip (Tulipa gesneriana L.)	*pollen*	*BR (1)*	*unspecified*	*Abe, 1991*[3]
Typhaceae				
Broadleaf cattail (Typha latifolia)	*pollen*	*BR (2)*	*68*	*Schneider et al., 1983*[3]*; Abe, 1991*[3]
Dicotyledons – Apetalae				
Betulaceae				
Common alder (Alnus glutinosa (L.))	*pollen*	*BL, CS*	*unspecified*	*Plattner et al., 1986*
Cannabaceae				
Hemp (Cannabis sativa L.)	*seeds*	*CS*	*600*	*Takatsuto et al., 1996b*
	seeds	*BR (1)*	*1800*	*Takatsuto et al., 1996b*
Caryophyllaceae				
Gypsophilla perfoliata L.	*seeds*	*24-epiBL*	*unspecified*	*Schmidt et al., 1996*
Sticky catchfly (Lychnis viscaria L.)	*seeds*	*24-epiCS, BR (1)*	*unspecified*	*Friebe et al., 1999*[3]
Chenophyllaceae				
Beet (Beta vulgaris L.)	*seeds*	*CS, 24-epiCS*	*unspecified*	*Schmidt et al., 1994*[3]
Fagaceae				
Japanese chestnut (Castanea crenata Sieb. Et Zucc.)	*galls*	*BL*	*0.001-12*	*Yokota et al., 1982a, Ikeda et al., 1983, Ikekawa & Takatsuto, 1984*
	galls	*CS*	*0.011-11.43*	*Yokota et al., 1982a, Ikeda et al., 1983, Ikekawa & Takatsuto, 1984*
	galls	*BR (2)*	*0.011-26*	*Yokota et al., 1982a, Ikeda et al., 1983, Ikekawa & Takatsuto, 1984*
	shoot	*BR (1)*	*15-30*	*Arima et al., 1984*
	leaves	*CS*	*2-6*	*Arima et al., 1984*
Polygonaceae				
Common buckwheat (Fagopyrum esculentum Moench)	*pollen*	*BL*	*5*	*Takatsuto et al., 1990b*
	pollen	*CS*	*7.1*	*Takatsuto et al., 1990b*
	grains	*24-epiBL*	*378*	*Khripach et al, 2013*
	grains	*BL*	*40*	*Khripach et al, 2013*
	grains	*28-homoBL*	*8.1*	*Khripach et al, 2013*
Pieplant (Rheum rhabarbarum L.)	*panicles*	*BL, CS, 24-epiCS*	*unspecified*	*Schmidt et al., 1995a*[3]

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
Dicotyledons – Chloripetalae				
Apiaceae				
Asian pennywort (Centella asiatica)	*leaves*	*CS*	*unspecified*	*Sondhi et al., 2010*
Celery (Apium graveolens L.)	*seeds*	*BR (1)*	*unspecified*	*Schmidt et al., 1995c*[3]
Wild carrot (Daucus carota ssp. Sativus L.)	*seeds*	*BL, CS, 24-epiCS*	*unspecified*	*Schmidt et al., 1998*[3]
	root	*24-epiBL*	*0.43*	*Khripach et al., 2013*
	root	*BL*	*1.5*	*Khripach et al., 2013*
	root	*28-homoBR*	*0.83*	*Khripach et al., 2013*
	root	*24-epiCS*	*0.23*	*Khripach et al., 2013*
	whole plant	*24-epiBL*	*0.745*	*Swaczynová et al., 2007*
	whole plant	*BL*	*0.644*	*Swaczynová et al., 2007*
	whole plant	*CS*	*0.316*	*Swaczynová et al., 2007*
	whole plant	*24-epiCS*	*0.642-1.19*	*Swaczynová et al., 2007*
Brassicaceae				
Arabidopsis thaliana (L.) Heynh.	*shoot*	*BL*	*0.04*	*Fujioka et al., 1996, 1997, 2000a*[3]*; Nomura et al., 2001*
	shoot	*CS*	*0.75*	*Fujioka et al., 1996, 1997, 2000a*[3]*; Nomura et al., 2001*
	shoot	*BR (9)*	*0.025-1.96*	*Fujioka et al., 1996, 1997, 2000a*[3]*; Nomura et al., 2001*
	20-days-old shoots	*BR (5)*	*0.1-0.79*	*Bancos et al., 2002*
	20-days-old shoots	*CS*	*0.15*	*Bancos et al., 2002*
	seeds	*24-epiBL*	*0.22*	*Fujioka et al., 1998*[3]
	seeds	*BL*	*0.5-1.9*	*Fujioka et al., 1998*[3]
	seeds	*CS*	*0.4-5*	*Fujioka et al., 1998*[3]
	seeds	*BR (4)*	*0.5-5.4*	*Fujioka et al., 1998*[3]
	seeds	*24-epiBL*	*0.22*	*Schmidt et al., 1997*
	seeds	*CS*	*0.36*	*Schmidt et al., 1997*
	root callus	*BL, BR (1)*	*unspecified*	*Konstantinova et al., 2001*[3]
	20-days-old roots	*BR (5)*	*0.09-1.8*	*Bancos et al., 2002*
	20-days-old roots	*CS*	*0.035*	*Bancos et al., 2002*
	seedlings	*BR (10)*	*unspecified*	*Choe et al., 2001 ; Fujioka et al. 2002*
	whole plant	*24-epiBL*	*3.634-4.566*	*Swaczynová et al., 2007*
	whole plant	*BL*	*1.245*	*Swaczynová et al., 2007*
	whole plant	*CS*	*0.562*	*Swaczynová et al., 2007*
	apical shoot	*BR (6)*	*0.03-7.93*	*Shimada et al., 2003*
	apical shoot	*CS*	*2.02*	*Shimada et al., 2003*
	stem	*BR (5)*	*0.14-2.64*	*Shimada et al., 2003*
	stem	*CS*	*0.40*	*Shimada et al., 2003*
	cauline leaves	*BR (5)*	*0.11-4.33*	*Shimada et al., 2003*
	cauline leaves	*CS*	*0.31*	*Shimada et al., 2003*
	rosette leaves	*BR (5)*	*0.06-2.85*	*Shimada et al., 2003*
	rosette leaves	*CS*	*0.13*	*Shimada et al., 2003*
	silique	*BR (5)*	*0.36-8.89*	*Shimada et al., 2003*
	silique	*CS*	*0.94*	*Shimada et al., 2003*
Chinese Cabbage (Brassica	*seeds*	*BL*	*940*	*Abe et al., 1982, 1983*[3]*;*

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
campestris var. pekinensis L.)				*Ikekawa et al., 1984*[3]
	seeds	*CS*	*1600*	*Abe et al., 1982, 1983*[3]*; Ikekawa et al., 1984*[3]
	seeds	*28-homoCS*	*130*	*Abe et al., 1982, 1983*[3]*; Ikekawa et al., 1984*[3]
	seeds	*BR (2)*	*780-1300*	*Abe et al., 1982, 1983*[3]*; Ikekawa et al., 1984*[3]
	immature seeds and sheaths	*BL*	*0.0094*	*Ikekawa & Takatsuto, 1984*
	immature seeds and sheaths	*CS*	*0.0016*	*Ikekawa & Takatsuto, 1984*
	immature seeds and sheaths	*BR (3)*	*0.0013-0.00078*	*Ikekawa & Takatsuto, 1984*
Indian mustard (Brassica juncea L.)	*fresh leaves*	*24-epiBL*	*unspecified*	*Kanwar et al., 2013*
Oilseed rape (Brassica napus L.)	*pollen*	*BL*	*100*	*Grove et al., 1979*
	breaking wall pollen	*24-epiBL*	*628*	*Pan et al., 2012*
	pollen	*BL*	*101.664*	*Swaczynová et al., 2007*
	pollen	*CS*	*12.166*	*Swaczynová et al., 2007*
Radish (Raphanus sativus L.)	*seeds*	*BL*	*0.3*	*Schmidt et al., 1991*[3]*, 1993b*[3]
	seeds	*CS*	*0.8*	*Schmidt et al., 1991*[3]*, 1993b*[3]
	seeds	*BR (2)*	*unspecified*	*Schmidt et al., 1991*[3]*, 1993b*[3]
	germinated seeds	*BL*	*0.45*	*Schmidt et al., 1991*[3]
	germinated seeds	*CS*	*0.4*	*Schmidt et al., 1991*[3]
Fabaceae				
Lablab bean (Dolichos lablab L.)	*seeds*	*BR (4)*	*12-160*	*Baba et al., 1983; Yokota et al., 1982b*[3]*, 1983b, 1984*
	seeds	*BL, CS, BR (2)*	*unspecified*	*Baba et al., 1983; Yokota et al., 1982b*[3]*, 1983b, 1984*
Dolichos lablab	*immature seeds*	*Homodolicholide*	*0.353*	*Yokota et al., 1983b*
False acacia (Robinia pseudo-acacia)	*pollen*	*CS, BR (2)*	*unspecified*	*Abe et al., 1995b*
Broad bean (Vicia faba L.)	*pollen*	*24-epiBL*	*5*	*Park et al., 1987; Ikekawa et al., 1988*
	pollen	*BL*	*190*	*Park et al., 1987; Ikekawa et al., 1988*
	pollen	*CS, BR (1)*	*unspecified*	*Park et al., 1987; Ikekawa et al., 1988*
	pollen	*BL*	*181*	*Gamoh et al., 1989*[3]
	pollen	*CS*	*134*	*Gamoh et al., 1989*[3]
	pollen	*BR (2)*	*537-628*	*Gamoh et al., 1989*[3]
Serradella (Ornithopus sativus Brot.)	*seeds*	*CS*	*5*	*Schmidt et al., 1993a*[3]
	seeds	*24-epiCS*	*25*	*Schmidt et al., 1993a*[3]
	shoot	*CS, 24-epiCS, BR (3)*	*unspecified*	*Spengler et al., 1995*[3]
Common bean (Phaseolus vulgaris L.)	*seeds*	*24-epiCS, BL, CS, BR (22)*	*unspecified*	*Yokota et al., 1983c, 1987 ; Kim et al., 1987, 1988*[3]*, 2000b ; Kim, 1991 ; Park et al., 2000*
	10-day-old seedlings	*24-epiBL*	*<0.346*	*Swaczynová et al., 2007*
	10-day-old	*BL*	*0.471*	*Swaczynová et al., 2007*

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
	seedlings			
	10-day-old seedlings	*CS*	*0.967*	*Swaczynová et al., 2007*
Goa bean (Psophocarpus tetragonolobus (Stickm.) DC.)	*seeds*	*BL, CS, BR (2)*	*unspecified*	*Takatsuto, 1994*[3]
Pea (Pisum sativum L.)	*seeds*	*BL, CS, BR (3)*	*unspecified*	*Yokota et al., 1996*[3]
	shoot	*BL*	*0.2-0.8*	*Nomura et al., 1997, 1999, 2001*
	shoot	*CS*	*0.4-2.4*	*Nomura et al., 1997, 1999, 2001*
	shoot	*BR (6)*	*0.047-5.2*	*Nomura et al., 1997, 1999, 2001*
	15- days-old shoots	*BR (5)*	*0.073-11.7*	*Bancos et al., 2002*
	15- days-old shoots	*CS*	*0.69*	*Bancos et al., 2002*
	shoots (36 d old)	*BL*	*0.164*	*Nomura et al., 1997*
	shoots (36 d old)	*CS*	*0.355*	*Nomura et al., 1997*
	shoots (36 d old)	*BR (1)*	*3.133*	*Nomura et al., 1997*
	6 months old plants	*BL*	*0.84*	*Nomura et al., 1997*
	6 months old plants	*CS*	*2.36*	*Nomura et al., 1997*
	6 months old plants	*BR (1)*	*0.995*	*Nomura et al., 1997*
	49-d-old shoots	*CS*	*0.491*	*Nomura et al., 1999*
	49-d-old shoots	*BR (7)*	*0.02-2.937*	*Nomura et al., 1999*
	15- days-old roots	*BR (6)*	*0.002-5.1*	*Bancos et al., 2002*
	15- days-old roots	*BL*	*0.024*	*Bancos et al., 2002*
	15- days-old roots	*CS*	*0.038*	*Bancos et al., 2002*
Hamamelidaceae				
Distylium racemosum Sieb. Et Zucc.	*galls*	*CS*	*2500*	*Ikekawa et al., 1984*[3]
	galls	*BR (1)*	*5*	*Ikekawa et al., 1984*[3]
	leaves	*BL*	*0.023*	*Ikekawa et al., 1984*[3]*, Abe et al., 1994*
	leaves	*CS*	*0.13*	*Ikekawa et al., 1984*[3]*, Abe et al., 1994*
	leaves	*BR(4)*	*0.016-0.16*	*Ikekawa et al., 1984*[3]*, Abe et al., 1994*
Myrtaceae				
Eucalyptus calophylla R. Br.	*pollen*	*BL*	*unspecified*	*Takatsuto, 1994*[3]
Eucalyptus marginata Sn.	*pollen*	*BR (1)*	*unspecified*	*Takatsuto, 1994*[3]
Rosaceae				
Loquat (Eriobotyra japonica (Thunb.) Lindl.)	*flower, buds*	*CS*	*unspecified*	*Takatsuto, 1994*[3]
Apple (Malus domestica)	*fruit*	*24-epiBL*	*27*	*Khripach et al, 2013*
	fruit	*BL*	*35*	*Khripach et al, 2013*
	fruit	*28-homoBL*	*10*	*Khripach et al, 2013*
Rutaceae				

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
Bael tree (Aegle marmelos Corr.)	*leaves*	*24-epiBL*	*unspecidied*	*Sondhi et al., 2008*
Satsuma orange (Citrus unshiu Marcov.)	*pollen*	*BL, CS, BR (2)*	*unspecified*	*Abe, 1991*[3]
Orange (Citrus sinensis Osbeck)	*pollen*	*BL*	*36.2*	*Motegi et al., 1994*
	pollen	*CS*	*29.4*	*Motegi et al., 1994*
Theaceae				
Chinese Tea (Thea sinensis L.)	*leaves*	*BL*	*0.006*	*Abe et al. 1983*[3]*, 1984a; Morishita et al., 1983*[3]*; Ikekawa et al., 1984*[3]
	leaves	*CS*	*0.1*	*Abe et al. 1983*[3]*, 1984a; Morishita et al., 1983*[3]*; Ikekawa et al., 1984*[3]
	leaves	*BR (4)*	*<0.001-0.06*	*Abe et al. 1983*[3]*, 1984a; Morishita et al., 1983*[3]*; Ikekawa et al., 1984*[3]
	seeds	*BR (6)*	*unspecified*	*Kaur et al., 2002*[3]
Green tea	*leaves*	*24-epiBL*	*100*	*Khripach et al, 2013, Gupta et al., 2004*
	leaves	*BL*	*0.0046*	*Ikekawa & Takatsuto, 1984*
	leaves	*CS*	*0.11*	*Ikekawa & Takatsuto, 1984*
	leaves	*BR (6)*	*0.002*	*Ikekawa & Takatsuto, 1984, Gupta et al., 2004*
Dicotyledons – Sympetalae				
Apocynaceae				
Catharanthus roseus G. Don.	*culture cells*	*BL*	*0.4-8.7*	*Choi et al., 1993, 1996*[3]*, 1997*[3] *; Fujioka et al., 1995, 2000b*[3] *; Park et al., 1989 ; Suzuki et al., 1993*[3]*, 1994a*[3]*, c, 1995 ; Yokota et al., 1990 ; Choe et al., 2001 ; Fujioka et al., 2002*
	culture cells	*CS*	*0.6-4.5*	*Choi et al., 1993, 1996*[3]*, 1997*[3] *; Fujioka et al., 1995, 2000b*[3] *; Park et al., 1989 ; Suzuki et al., 1993*[3]*, 1994a*[3]*, c, 1995 ; Yokota et al., 1990 ; Choe et al., 2001 ; Fujioka et al., 2002*
	culture cells	*BR (17)*	*0.047-30*	*Choi et al., 1993, 1996*[3]*, 1997*[3] *; Fujioka et al., 1995, 2000b*[3] *; Park et al., 1989 ; Suzuki et al., 1993*[3]*, 1994a*[3]*, c, 1995 ; Yokota et al., 1990 ; Choe et al., 2001 ; Fujioka et al., 2002*
Asteraceae				
Common sunflower (Helianthus anuus L.)	*pollen*	*BL*	*106*	*Takatsuto et al., 1989*
	pollen	*CS*	*21*	*Takatsuto et al., 1989*
	pollen	*BR (1)*	*65*	*Takatsuto et al., 1989*
	breaking wall pollen	*24-epiBL*	*1930*	*Pan et al., 2012*
Solidago altissima L.	*shoot*	*BL*	*unspecified*	*Takatsuto, 1994*[3]
Zinnia elegans L.	*culture cells*	*CS, BR (4)*	*unspecified*	*Yamamoto et al., 2001*
Boraginaceae				
Echium plantagineum L.	*pollen*	*BL*	*unspecified*	*Takatsuto, 1994*[3]
Convolvulaceae				
Pharbitis purpurea Voigt	*seeds*	*CS*	*1.1*	*Suzuki et al., 1985*
	seeds	*BR (1)*	*0.2*	*Suzuki et al., 1985*

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
Cucurbitaceae				
Cucurbita moschata Duch.	*seeds*	*BL, CS*	*unspecified*	*Jang et al., 2000*
Lamiaceae				
Perilla frutescens (L.) Britt.	*seeds*	*CS*	*unspecified*	*Park et al., 1994b*
Plantaginaceae				
Coastal water hyssop (Bacopa monnieri L.)	*Fresh leaves*	*24-epiBL*	*unspecified*	*Tripathi & Sharma, 2015*
Rubiaceae				
Coffee (Coffea arabica)	*bean*	*24-epiBL*	*30*	*Khripach et al., 2013*
	bean	*BL*	*250*	*Khripach et al., 2013*
	bean	*28-homoBL*	*23*	*Khripach et al., 2013*
Solanaceae				
Tobacco (Nicotiana tabacum L.)	*culture cells*	*CS*	*unspecified*	*Park et al., 1994b*
Tomato (Lycopersicum esculentum Mill.)	*shoot*	*CS*	*0.2*	*Yokota et al., 1997d*
	shoot	*BR (2)*	*0.03-1.7*	*Yokota et al., 1997d*
	shoot (dwarf mutant)	*BL*	*<0.001*	*Bishop et al., 1999*
	shoot (dwarf mutant)	*CS*	*0.2*	*Bishop et al., 1999*
	shoot (dwarf mutant)	*BR (10)*	*<0.001-52*	*Bishop et al., 1999*
	36- days-old shoots	*BR (5)*	*0.016-0.64*	*Bancos et al., 2002*
	36- days-old shoots	*CS*	*0.14*	*Bancos et al., 2002*
	36- days-old roots	*BR (5)*	*0.062-2.8*	*Bancos et al., 2002*
	36- days-old roots	*CS*	*0.011*	*Bancos et al., 2002*
Potato (Solanum tuberosum)	*tuber*	*24-epiBL*	*37.5*	*Khripach et al., 2013*
	tuber	*BL*	*10*	*Khripach et al., 2013*
	tuber	*28-homoBL*	*1.5*	*Khripach et al., 2013*
	tuber	*Epi-CS*	*1.7*	*Khripach et al., 2013*
Gymnosperms				
Cupressaceae				
Cupressus arizonica Greene	*pollen*	*BL*	*<1*	*Griffiths et al., 1995*
	pollen	*CS*	*1000*	*Griffiths et al., 1995*
	pollen	*BR (7)*	*2-6400*	*Griffiths et al., 1995*
Ginkgoaceae				
Ginkgo biloba L.	*seeds*	*BR (1)*	*15*	*Takatsuto et al., 1996a*
Pinaceae				
Picea sitchensis Trantv. ex Mey	*shoot*	*CS*	*5*	*Yokota et al., 1985*[3]
	shoot	*BR (1)*	*7*	*Yokota et al., 1985*[3]
Pinus silvestris L.	*cambial region*	*BL, CS*	*unspecified*	*Kim et al., 1990*
Pinus thunbergii Parl.	*pollen*	*BR (1)*	*89.5*	*Yokota et al., 1983a*
Taxodiaceae				
Crypromeria japonica D. Don.	*pollen, anther*	*28-homoBL, BR (8)*	*unspecified*	*Yokota et al., 1998, Watanabe et al., 2000*
Lower plants				
Athyriaceae				

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
Black lady fern (Deparia japonica)	*fertile frond*	*CS*	*0.008*	*Yokota et al., 2017*
	fertile frond	*BR (7)*	*0.013-4.867*	*Yokota et al., 2017*
Asian common ladyfern (Athyrium yokoscense)	*reproductive frond*	*CS*	*0.002*	*Yokota et al., 2017*
	reproductive frond	*BR (6)*	*0.073-4.807*	*Yokota et al., 2017*
Dennstaedtiaceae				
Eagle fern (Pteridium aquilinum)	*vegetative frond*	*CS*	*0.003*	*Yokota et al., 2017*
	vegetative frond	*BR (7)*	*0.021-1.873*	*Yokota et al., 2017*
Dryopteridaceae				
Wood fern (Dryopteris crassirhizoma)	*fertile frond*	*CS*	*0.024*	*Yokota et al., 2017*
	fertile frond	*BR (3)*	*0.019-0.802*	*Yokota et al., 2017*
Autumn fern (Dryopteris erythrosora)	*reproductive shoot*	*CS*	*0.005*	*Yokota et al., 2017*
	reproductive shoot	*BR (6)*	*0.008-20.87*	*Yokota et al., 2017*
Cyrtomium laetevirens	*reproductive shoot*	*CS*	*0.002*	*Yokota et al., 2017*
	reproductive shoot	*BR (5)*	*0.006-3.172*	*Yokota et al., 2017*
Equisetaceae				
Field Horsetail (Equisetum arvense L.)	*whole plant*	*CS*	*0.17*	*Takatsuto et al., 1990a*
	whole plant	*BR (3)*	*0.15-0.75*	*Takatsuto et al., 1990a*
	shoot	*CS*	*0.003-0.008*	*Yokota et al., 2017*
	shoot	*BR (8)*	*0.02-2*	*Yokota et al., 2017*
Funariaceae				
Spreading earth-moss (Physcomitrella patens)	*protonema*	*CS*	*0.004*	*Yokota et al., 2017*
	protonema	*BR (8)*	*0.008-1.122*	*Yokota et al., 2017*
Lygodiaceae				
Vine-like fern (Lygodium japonicum)	*vegetative frond*	*CS*	*0.016*	*Yokota et al., 2017*
	vegetative frond	*BR (7)*	*0.005-25.41*	*Yokota et al., 2017*
Marchantiaceae				
Common liverwort (Marchantia polymorpha L.)	*culture cells*	*BR (3)*	*unspecified*	*Park et al., 1999*
	thallus	*CS*	*0.006-0.038*	*Yokota et al., 2017*
	thallus	*BR (6)*	*0.001-0.139*	*Yokota et al., 2017*
	on agar medium	*CS*	*0.007*	*Yokota et al., 2017*
	on agar medium	*BR (5)*	*0.002-0.119*	*Yokota et al., 2017*
Onocleaceae				
Bead fern (Onoclea sensibilis)	*vegetative frond*	*CS*	*0.003*	*Yokota et al., 2017*
	vegetative frond	*BR (3)*	*0.063-0.19*	*Yokota et al., 2017*
Fiddlehead fern (Matteuccia struthiopteris)	*vegetative frond*	*CS*	*0.016*	*Yokota et al., 2017*
	vegetative frond	*BR (3)*	*0.15-1.175*	*Yokota et al., 2017*

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
Osmundaceae				
Asian royal fern (Osmunda japonica)	*vegetative frond*	*CS*	*0.004-0.005*	*Yokota et al., 2017*
	vegetative frond	*BR (11)*	*0.007-202.9*	*Yokota et al., 2017*
Selaginellaceae				
Spikemoss (Selaginella moellendorffii)	*frond*	*CS*	*0.02*	*Yokota et al., 2017*
	frond	*BR (2)*	*<0.042-0.084*	*Yokota et al., 2017*
Blue Spikemoss (Selaginella uncinata)	*frond*	*CS*	*0.006*	*Yokota et al., 2017*
	frond	*BR (6)*	*0.007-0.275*	*Yokota et al., 2017*
Thelypteridaceae				
Japanese Beech Fern (Thelypteris decursive-pinnata)	*fertile frond*	*CS*	*0.015*	*Yokota et al., 2017*
	fertile frond	*BR (7)*	*0.025-5.119*	*Yokota et al., 2017*
Marsh fern (Thelypteris palustris)	*vegetative frond*	*BR (6)*	*0.002-1.122*	*Yokota et al., 2017*
Algae				
Chaetophoraceae				
Green algae (Stigeoclonium nanum)	*cultured cells*	*BL*	*168.7 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*144.9 µg/kg dr. wt*	*Stirk et al., 2013*
Chlamydomonadaceae				
Chlamydomonas reinhardtii	*cultured cells*	*BL*	*162.9 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*153.8 µg/kg dr. wt*	*Stirk et al., 2013*
Protococcus viridis	*cultured cells*	*BL*	*211.6 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*134.8 µg/kg dr. wt*	*Stirk et al., 2013*
Chlamydomonadaceae				
	cultured cells	*BL*		*Stirk et al., 2013*
Chlorellaceae				
Green algae (Clorella vulgaris)	*cultured cells*	*BL*	*0.07*	*Bajguz, 2009*
Green algae (Clorella vulgaris)	*cultured cells*	*CS*	*0.47*	*Bajguz, 2009*
Green algae (Clorella vulgaris)	*cultured cells*	*BR (5)*	*0.18-0.39*	*Bajguz, 2009*
Green algae (Clorella pyrenoidosa)	*cultured cells*	*BL*	*253 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*158 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae (Clorella vulgaris)	*cultured cells*	*BL*	*193.3 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*215.3 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae (Clorella minutissima)	*cultured cells*	*BL*	*306.5 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*215.3 µg/kg dr. wt*	*Stirk et al., 2013*
Chlorococcaceae				
Green algae Chlorococcum ellipsoideum	*cultured cells*	*BL*	*168.7 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*105.7 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae Nautococcus mamillatus	*cultured cells*	*BL*	*115.8 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*99.9 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae Spongiochloris excentrica	*cultured cells*	*BL*	*131.2 µg/kg dr. wt*	*Stirk et al., 2013*

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
	cultured cells	*CS*	*108.5 µg/kg dr. wt*	*Stirk et al., 2013*
Coccomyxaceae				
Green algae Coccomyxa sp.	*cultured cells*	*BL*	*205.8 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*177.1 µg/kg dr. wt*	*Stirk et al., 2013*
Hydrodictyaceae				
Green algae (Hydrodictyon reticulatum (L.) Lager	*cultured cells*	*24-epiCS*	*0.3*	*Yokota et al., 1987b*[3]
	cultured cells	*28-homoCS*	*4*	*Yokota et al., 1987b*[3]
Klebsormidiaceae				
Green algae (Klebsormidium flaccidum)	*cultured cells*	*BL*	*548.7 µg/kg dr. wt*	*Stirk et al., 2013*
		CS	*429.1 µg/kg dr. wt*	*Stirk et al., 2013*
Neochloridaceae				
Green algae (Poloidion didymos)	*cultured cells*	*BL*	*167.3 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*172.8 µg/kg dr. wt*	*Stirk et al., 2013*
Palmellaceae				
Green algae (Gyoerffyana humicola)	*cultured cells*	*BL*	*270.9 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*201.1 µg/kg dr. wt*	*Stirk et al., 2013*
Prasiolaceae				
Green algae (Stichococcus bacillaris)	*cultured cells*	*BL*	*291.8 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*242.7 µg/kg dr. wt*	*Stirk et al., 2013*
Protosiphonaceae				
Green algae (Protosiphon botryoides)	*cultured cells*	*BL*	*100.6 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*74 µg/kg dr. wt*	*Stirk et al., 2013*
Scenedesmaceae				
Green algae (Acutodesmus acuminatus)	*cultured cells*	*BL*	*125.1 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*105.5 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae (Acutodesmus incrassatulus)	*cultured cells*	*BL*	*124.8 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*92.6 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae (Desmodesmus armatus)	*cultured cells*	*BL*	*125.1 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*109.3 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae (Scotiellopsis terrestris)	*cultured cells*	*BL*	*336.9 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*235.9 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae (Coelastrum microporum)	*cultured cells*	*BL*	*199.2 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*158.3 µg/kg dr. wt*	*Stirk et al., 2013*
Selenastraceae				
Green algae (Monoraphidium contortum)	*cultured cells*	*BL*	*284.9 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*195 µg/kg dr. wt*	*Stirk et al., 2013*
Green algae (Raphidocelis subcapitata)	*cultured cells*	*BL*	*58.6 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*58.7 µg/kg dr. wt*	*Stirk et al., 2013*
Trebouxiaceae				
Green algae (Myrmecia bisecta)	*cultured cells*	*BL*	*202.4 µg/kg dr. wt*	*Stirk et al., 2013*

Family/Species	*Examined part*	*Brassino-steroid*[1]	*content µg/kg fr. wt.*[2]	*References*
	cultured cells	*CS*	*164.3 µg/kg dr. wt*	*Stirk et al., 2013*
Ulotrichaceae				
Green algae (Ulothrix sp.)	*cultured cells*	*BL*	*84.9 µg/kg dr. wt*	*Stirk et al., 2013*
	cultured cells	*CS*	*74.2 µg/kg dr. wt*	*Stirk et al., 2013*
Crystoseiraceae				
Brown algae (Cystoseira myrica (Gmelin) Agardh)	*whole plant*	*BR*	*unspecified*	*Hamdy et al., 2009*
Fungi				
Cercospora arachidicola	*unspecified*	*unspecified*	*unspecified*	*Zakharychev, 1999*[3] *in Tsavkelova et al., 2006*
Processed foods				
Juice and Wines				
Apple juice	*juice*	*24-epiBL*	*12*	*Khripach et al., 2013*
Apple juice	*juice*	*BL*	*1.7*	*Khripach et al., 2013*
Apple juice	*juice*	*28-homoBL*	*3*	*Khripach et al., 2013*
Grape juice	*juice*	*24-epiBL*	*1.7*	*Khripach et al., 2013*
Grape juice	*juice*	*BL*	*1.8*	*Khripach et al., 2013*
Grape juice	*juice*	*28-homoBL*	*0.4*	*Khripach et al., 2013*
Pineapple juice	*juice*	*24-epiBL*	*3*	*Khripach et al., 2013*
Pineapple juice	*juice*	*BL*	*1.6*	*Khripach et al., 2013*
Pineapple juice	*juice*	*28-homoBL*	*0.5*	*Khripach et al., 2013*
Birch juice	*juice*	*24-epiBL*	*0.5*	*Khripach et al., 2013*
Birch juice	*juice*	*BL*	*1.2*	*Khripach et al., 2013*
Birch juice	*juice*	*28-homoBL*	*0.1*	*Khripach et al., 2013*
Dry red wine (Merlot)	*wine*	*24-epiBL*	*3*	*Khripach et al., 2013*
Dry red wine (Merlot)	*wine*	*BL*	*10*	*Khripach et al., 2013*
Dry red wine (Merlot)	*wine*	*28-homoBL*	*4.2*	*Khripach et al., 2013*
Honey				
Honey		*24-epiBL*	*7.4*	*Khripach et al., 2013*
Honey		*BL*	*1*	*Khripach et al., 2013*

[1] *24-epiBL = 24-Epibrassinolide; 24-epiCS=24-Epicastasterone (precursor of 24-Epibrassinolide); BL = Brassinolide; CS=Castasterone (precursor of Brassinolide); 28-homoBL = 28-Homobrassinolide; 28-homoCS = 28-Homocastasterone (precursor of 28-Homobrassinolide); BR (Nr.)= Other Brassinosteroids (Number)*

[2] *Amount of Brassinosteriod is expressed in µg/kg fresh weight, if not specified otherwise*

[3] *Cited in the review publications Bajguz and Tretyn (2003) and Hayat and Ahmad (2011).*

24-Epibrassinolide elicits and activates the plant's self-defence mechanisms mediating the plant's resistance to unfavourable environmental conditions, (e.g. salinity, drought, cold and heat stress) and fungal diseases.

Application of brassinosteroids leads to a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, induction of protein synthesis and the production of various chemical defence compounds (Bajguz and Hayat, 2009). Brassinosteroid treated plants are not only more tolerant to biotic but also to abiotic stresses, providing a solution for problems that could arise in agriculture in the course of the climate change (Eremina et al., 2016).

Humans are constantly exposed to 24-Epibrassinolide through consumption of plants and plant organs, e.g. seeds, roots, and leafs (0.22 - 378 µg/kg), as well as other natural and processed foods such as honey (7.4 µg/kg), fruit juices (0.5 - 12 µg/kg) and wine (3 µg/kg) (Table A-2) and thus 24-Epibrassinolide has no relevant toxicity hazard towards humans.

EFSA (2012) has even concluded that plant sterols (which includes 24-Epibrassinolide) are not only of low risk for the human consumer but necessary for a healthy diet as they are contributing to lowering the LDL-

cholesterol levels, which is pivotal for the prevention of coronary heart diseases. Therefore, a daily intake of up to 3 g of plant sterols per day is highly recommended by EFSA (see CA 5.9.2).

Brassinosteroids are also non-toxic to non-target organisms. Mammals, aquatic organisms, insects, and soil organisms are constantly exposed to Brassinosteroids through the consumption of Brassinosteroids contained in higher and lower plants (present in soil, fresh- and seawater). Furthermore, no effects on soil microorganisms are expected. Not only are certain soil microorganisms able to metabolize Brassinosteroids, but some microorganisms are also able to synthesize Brassinosteriods themselves (Tsavkelova et al., 2006).

Non-target soil organisms are constantly exposed to Brassinosteroids, not only from the constant release of Brassinosteroid from decaying plant material (e.g. Aremu et al., 2015) but also from the Brassinosteroid precursors, campesterol, sitosterol, and stigmasterol. These precursors are known root exudates and are involved in the mediation of interactions in the rhizosphere, which includes the symbiotic associations with beneficial microbes, such as mycorrhizae, rhizobia, and plant growth-promoting rhizobacteria (PGPR) (Badri and Vivanco, 2009).

Due to the constant formation and decomposition of plant root systems, the presence of seeds, pollen, and decomposing plant material and the release of Brassinosteroids from decomposing organic matter (e.g. Aremu et al., 2015) as well as the vast number of other Brassinosteroid producing organisms such as algae in the environment, Brassinosteroids – and other pytosterols – are naturally present in all environmental compartments including soil e.g. Aremu et al., 2015) and water-bodies including sediment (Hassett & Lee, 1977; Mudge et al., 1999).

In addition to that, bioaccumulation is not expected as Brassinosteroids are readily absorbed and metabolised by higher and lower plants (e.g. Nishikawa et al., 1994), diatoms (e.g. Mekhalfi et al., 2012), green algae (e.g. Bajguz, 2011), fungi (e.g. Voigt et al., 1993), mycobacteria (e.g. Vorbrot et al., 1991), and cyanobacteria (e.g. Saygideger and Deniz, 2008). As Brassinosteroids are phylogenetically ancient phytohormones, it can be expected that each organism has developed its own co-evolutionary mechanism to metabolise these phytohormones. It was further found that Brassinosteroid synthesis in plants is naturally triggered for example by microorganisms (Asari et al., 2017).

24-Epibrassinolide can be considered as low risk active substance in accordance with Regulation (EC) 1107/2009, Annex II point 5, as it is not classified as carcinogenic, mutagenic, toxic to reproduction, sensitising, very toxic or toxic, explosive or corrosive and it is not considered persistent, bio-accumulating, endocrine disrupting or neuro- or immunotoxic. Further, it fulfils all low risk criteria indicated in the draft working documents for the purpose of a possible amendment of the current low-risk criteria (Sante/xxxxx/2015 rev. 2, July 2015). In addition, it is a natural, ubiquitous occurring plant molecule, which is expected to have no negative effects on the environment, non-target organism or humans.

24-Epibrassinolide has a very low toxicity profile and is ubiquitous distributed in the plant kingdom (please see Table A-2) and therefore fulfils criterion 3 of SANCO/11188/2013 Rev. 2 of 14 September 2015: "The compound has no identified hazardous properties". In addition, criterion 4 of SANCO/11188/2013 Rev. 2 of 14 September 2015: "Natural exposure is higher than the one linked to the use as PPP", is met. Therefore, inclusion into Annex IV of Regulation (EC) N° 396/2005 is requested, as no maximum residue levels are required.

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