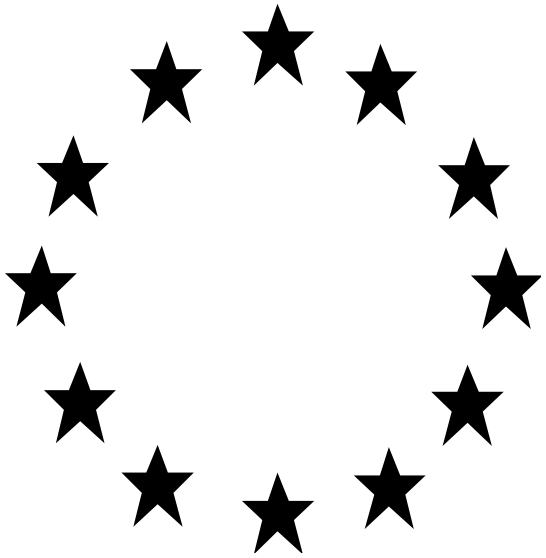

Draft Assessment Report (DAR)

- public version -

Initial risk assessment provided by the rapporteur Member State Estonia for the existing active substance

PHLEBIOPSIS GIGANTEA

of the fourth stage of the review programme referred to in Article 8(2) of Council Directive 91/414/EEC

Volume 3, Annex B, part 1, B.1 – B.5

September 2008

Draft Assessment Report

Phlebiopsis gigantea

Volume 3

Annex B.1

Identity

Rapporteur Member State: Estonia

April 2007

TAIMETOODANGU INSPEKTSIOON

Volume 1

Level 1: Statement of subject matter and purpose for which the monograph was prepared

Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Appendix 3: List of endpoints

Level 3: Proposed decision with respect to the application for inclusion of the active substance in Annex I

Level 4: Further information to permit a decision to be made, or to support a review of the conditions and restrictions associated with the proposed inclusion in Annex 1

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Annex A: List of the tests and studies submitted and of information available

Volume 3

Annex B: RMS summary, evaluation and assessment of the data and information

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Annex B.2: Biological, physical, chemical and technical properties

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B.1 Identity

B.1.1 Identity of the Micro-organism (Annex IIB 1)

B.1.1.1 Applicant (Annex IIB 1.1)

Name:	*Phlebiopsis gigantea* Task force (PGT)
Applicant:	Verdera Oy
	Verdera Oy
	Luoteisrinne 2
	P.O Box 5
	FI-02270 (FI-02271) ESPOO, FINLAND
Contact Point:	Ms. Marina Niemi
	Business development manager
	Phone: +358 10 217 3724
	Fax: +358 10 217 3711
	E-mail: marina.niemi@verdera.fi

B.1.1.2 Producer (Annex IIB 1.2)

B.1.1.2.1 Producer no. 1 of *Phlebiopsis gigantea*

Product:	Rotstop
Micro-organism:	*Phlebiopsis gigantea* (Fr.) Jülich
Strains:	VRA 1835
	VRA 1984
	VRA 1985
	VRA 1986
Manufacturer:	Verdera Oy
	Luoteisrinne 2
	P.O Box 5
	FI-02270 (FI-02271) ESPOO, FINLAND
Contact Point:	Mr. Pekka Seiskari
	Production Manager
	Phone: +358 10 217 3720
	Mobile phone: +358 50 330 4213
	Fax: +358 455 0907
	E-mail: pekka.seiskari@verdera.fi

B.1.1.2.2 Producer no. 2 of *Phlebiopsis gigantea*

Product:	PG Suspension
Micro-organism:	*Phlebiopsis gigantea* (Fr.) Jülich
Strains:	FOC PG B20/5
	FOC PG SP log 6
	FOC PG SP log 5
	FOC PG BU 3
	FOC PG BU 4
	FOC PG 410.3
	FOC PG97/1062/116/1.1
	FOC PG B22/SP1287/3.1
	FOC PG SH 1
	FOC PG B22/SP1190/3.2
Manufacturer:	Forest Research
	Alice Holt Lodge, Farnham
	Surrey GU10 4 LH, UNITED KINGDOM
Contact Point:	Dr. Katherine Thorpe
	Production Manager
	Phone: +44 1420 22255 ex 2241
	Fax: +44 1420 23653

B.1.1.3 Name and species description, strain characterisation (Annex IIB 1.3)

Species name: *Phlebiopsis gigantea*

Phlebiopsis gigantea (synonyms *Phlebia gigantea* (Fr) Donk, *Peniophora gigantea* (Fr.) Massee, *Phanerochaete gigantea* (Fries) Rattan *et al*.) is currently the most commonly used name for this fungus, although work on the precise taxonomic position of *P. gigantea* is on-going. However, until this question is finally resolved and the outcome generally accepted, the name *Phlebiopsis gigantea* is considered valid.

P. gigantea is a common and widely distributed saprophytic wood-decay fungus in the coniferous forests of the Northern Hemisphere. It is assumed to be ubiquitous in the whole of Europe. On the basis of its morphology, *P. gigantea* is regarded as a single taxonomic species throughout its geographical distribution. Pairing studies with *P. gigantea* isolates from different European countries have shown *P. gigantea* to be interfertile within Europe. Investigations of molecular markers have revealed some genetic variation among European *P. gigantea* populations, but the markers are equally distributed in strains from different locations, indicating low genetic differentiation. In contrast, genetic differences have been observed between European and North American populations. However, no clear indications of the existence of intersterility groups have been found, and they are regarded as belonging to the same biological species (Eriksson *et al*. 1978; Eriksson *et al*. 1981; Korhonen & Kauppila 1988; Vainio *et al*. 1998; Vainio &Hantula 2000; Parmasto & Hallenberg 2000; De Koker *et al*. 2003; Hollmer & Stenlid 2003; Webber & Thorpe 2003; Grillo *et al*. 2005).

There are 14 strains which are being notified.

B.1.1.3.1 Accession number in culture collection (Annex IIB 1.3.1)

The strains of *Phlebiopsis gigantea* are deposited in the American Type Culture Collection, in Deutsche Sammlung von Microoganismen und Zellculturen GmbH or in CABI Bioscience Safe deposit IMI. For accession numbers see table below:

Strain no.	Synonym	Culture collection accession no.	Reference
VRA 1835	KK910215.2.1	ATCC 90304	ATCC Accession form no. 90304 (1993)
VRA 1984	SLU D R 4.8	DSM 16201	DSMZ Accession form no. 16201 (2004)
VRA 1985	SLU C R 32.8	DSM 16202	DSMZ Accession form no. 16202 (2004)
VRA 1986	SLU E R 38.2	DSM 16203	DSMZ Accession form no. 16203 (2004)
FOC PG B20/5		IMI 390096	IMI Safe deposit certificate SD188 (2003)
FOC PG SP log 6	NRS Log 6	IMI 390097	IMI Safe deposit certificate SD189 (2003)
FOC PG SP log 5		IMI 390098	IMI Safe deposit certificate SD190 (2003)
FOC PG BU 3		IMI 390099	IMI Safe deposit certificate SD191 (2003)
FOC PG BU 4		IMI 390100	IMI Safe deposit certificate SD192 (2003)
FOC PG 410.3	PG 21 B22 Mull LP 410.3	IMI 390101	IMI Safe deposit certificate SD193 (2003)
FOC PG97/1062/116/1.1	97/1062/116 SP Buchan	IMI 390102	IMI Safe deposit certificate SD194 (2003)
FOC PG B22/SP1287/3.1	B22 SP 1287 (Inveramie)	IMI 390103	IMI Safe deposit certificate SD195 (2003)
FOC PG SH 1		IMI 390104	IMI Safe deposit certificate SD196 (2003)
FOC PG B22/SP1190/3.2		IMI 390105	IMI Safe deposit certificate SD197 (2003)

B.1.1.3.2 Scientific name and taxonomic grouping, *i.e.* family, genus, species, strain, serotype, pathovar or any other denomination relevant to the micro-organism (Annex IIB 1.3.2)

Kingdom	Fungi
Phylum:	Basidiomycota
Class:	Basidiomycetes
Subclass:	Agaricomycetidae
Order:	Polyporales
Family:	Phanerochaetaceae
Genus:	*Phlebiopsis*
Species:	*Phlebiopsis gigantea* (Fr.) Jülich

Phlebiopsis gigantea strains are indigenous wild type, not genetically modified, isolated from fruit bodies formed on *Picea* and *Pinus* stumps.

Strain no.	**Origin of isolate**	**Method of isolation, maintenance and initial testing**	**Culture collection number**
VRA 1835	Sporocarp on stump of *Picea abies* in Loppi, Finland	Single basidiospore and tissue isolations from the fruit body, culture stored on malt extract agar slants at 2-4 °C. Initial testing: growth rate on malt agar, control efficacy against *H. annosum* and *H. parviporum* in stumps and in log pieces of pine and spruce	ATTC 90304
VRA 1984	Sporocarp on stump of *Picea abies* in Uppsala Råberg, Sweden	Single basidiospore isolations from the fruit body, stored on Hagem agar plates. Initial testing*:* Visual assessment of spore abundance on Hagem agar, *in vitro* interactions between *P. gigantea* and *H. annosum* and *H. parviporum* respectively, i.e. overgrowth in dual culture on spruce discs, control efficacy in spruce stumps	DSM 16201
VRA 1985	Sporocarp on stump of *Picea abies* in Uppsala Råberg, Sweden	Single basidiospore isolations from the fruit body, stored on Hagem agar plates.. Initial testing*:* Visual assessment of spore abundance on Hagem agar, *in vitro* interactions between *P. gigantea* and *H. annosum* and *H. parviporum* respectively, i.e. overgrowth in dual culture on spruce discs, control efficacy in spruce stumps	DSM 16202
VRA 1986	Sporocarp on stump of *Picea abies* in Uppsala Råberg, Sweden	Single basidiospore isolations from the fruit body, stored on Hagem agar plates. Initaial testing*:* Visual assessment of spore abundance on Hagem agar, *in vitro* interactions between *P. gigantea* and *H. annosum* and *H. parviporum* respectively, i.e. overgrowth in dual culture on spruce discs, control efficacy in spruce stumps	DSM 16203
FOC PG B20/5	*Pinus sylvestris* stump, Roslin, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390096
FOC PG SP log 6	*Pinus sylvestris* stump, Roslin, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390097
FOC PG SP log 5	*Pinus sylvestris* stump, Roslin UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390098
FOC PG BU 3	*Pinus sylvestris* stump, Buchan, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390099
FOC PG BU 4	*Pinus sylvestris* stump, Buchan, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390100
FOC PG 410.3	*Pinus contorta* stump, Mull, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390101
FOC PG97/1062/116/1.1	*Pinus* species, unknown location, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390102
FOC PG B22/SP1287/3.1	*Pinus sylvestris* stump, unknown location, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390103

FOC PG SH 1	*Pinus nigra* var *laricio* stump, Sherwood, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390104
FOC PG B22/SP1190/3.2	*Pinus sylvestris* stump, unknown location, UK	Tissue isolations from the fruit body. Culture stored on malt agar slant at 4°C	IMI 390105

B.1.1.3.3 Test procedures and criteria used for identification (Annex IIB 1.3.3)

Identification on species level using classical taxonomy based on morphology and growth characteristics of the fungus (growth rate, microscopic appearance of mycelium and conidial structures, enzyme activity etc.). Identification on strain level based by using molecular identification methods e.g. RAPD and RAMS/PCR and strain-specific markers.

Phlebiopsis gigantea is morphologigally described in DSMZ study (Braunschweig, september 24, 2004): Colonies of *Phlebiopsis gigantea* are growing rapidly on malt-extract agar (Petri dish size 90 mm Ø, temperature 25 ° C, 5 days); mycelium hyaline, at first appressed, later farinaceous. Colony reverse pale: unchanged; laccase reaction with gualacol negative. No fruiting structures observed. Hyphal diameter variable (4-8 µm); no skeletal or binding hyphae; clamp connections present only on few submerged hyphae, seldom double or multiple. Aerial mycelium partially disintegrating to form numerous cylindrical arthrospores. Chiamydospores and other conidia absent.

Strain no.	**Molecular identification method**	**Morphological identification criteria**	**Reference**
VRA 1835	RAMS-PCR analysis	Growth characteristics of mycelium and spores. Enzyme activity.	Hallaksela & Korhonen (1992a) Hoffman (2004) Vainio *et al.* (2001)
VRA 1984	ITS-sequence analysis	Mycelial characteristics.	Korhonen (2003a) Holmer & Stenlid (2003)
VRA 1985	ITS-sequence analysis	Mycelial characteristics.	Korhonen (2003a) Holmer & Stenlid (2003)
VRA 1986	ITS-sequence analysis	Mycelial characteristics.	Korhonen (2003a) Holmer & Stenlid (2003)
FOC PG B20/5	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG SP log 6	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG SP log 5	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG BU 3	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG BU 4	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG 410.3	PCR-RAPD analysis	Growth characteristics of	Thorpe (2005a) Webber & Thorpe (2003)

		mycelium and spores	
FOC PG97/1062/116/1.1	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG B22/SP1287/3.1	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG SH 1	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG B22/SP1190/3.2	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)

B.1.1.3.4 Common name or alternative and superseded names and code names used during the development (Annex IIB 1.3.4)

Common names: White rot fungus (EN), Harmaaorvakka (FI), Pergamentsvamp (SE, FI), Stor barksopp (NO), Kempe barksvamp (DK), Suur korbik (EE)

Other names: *Phlebiopsis gigantea*, *Phlebia gigantea*, *Peniophora gigantea*, *P. gigantea,* P.g., PG,

Sometimes the product names Rotstop (= Rotstop F), Rotstop S and PG Suspension are used to refer to the active ingredient *P. gigantea*, although strictly these names refer to specific products.

B.1.1.3.5 Relationship to known pathogens (Annex IIB 1.3.5)

A database search was made using DIALINDEX (search term 'ALL SCIENCE') to investigate any reports of pathogenicity to humans and other mammals caused by species from family Phanerochaetaceae.

Of the genera grouped under Phanerochaeteceae there were no reports of deleterious impacts on human (or other animal) health. *Phlebiopsis gigantea* doesn't have any relationship to known pathogens.

B.1.1.4 Specification of the material used for manufacturing of formulated products (Annex IIB 1.4)

B.1.1.4.1 Content of the micro-organism (Annex IIB 1.4.1)

Technical Grade of the MPCA is a hypothetical stage in a continuous production process of end-use products with a strain of *P. gigantea* as active substance.

Depending on the type of formulation, the concentration of micro-organism in the formulated product is 2 x 10^6-10^7 cfu of *P. gigantea*/g (Rotstop) and 3.5 x10^6-10^7 cfu of *P. gigantea*/ml (PG Suspension).

B.1.1.4.2 Identity and content of impurities, additives, contaminating micro-organisms (Annex IIB 1.4.2)

The maximum accepted level for microbial impurities, typically mesophilic mould fungi, is 2 % of the viable count of *P gigantea* in the end-product.

For further details, see the Confidential information, Annex C.

B.1.1.4.3 Analytical profile of batches (Annex IIB 1.4.3)

Confidential information. See Annex C.

B.1.2 Identity of the plant protection product (Annex IIIB 1)

B.1.2.1 Applicant (Annex IIIB 1.1)

Product name:	Rotstop
Applicant:	Verdera Oy Riihitontuntie 14 A P.O Box 1 FI-02200 (FI-02201) ESPOO, FINLAND
Contact Point:	Ms. Marina Niemi Business development manager Phone: +358 10 217 3724 Fax: +358 10 217 3711 E-mail: marina.niemi@verdera.fi

B.1.2.2 Manufacturer of the preparation and the micro-organism (Annex IIIB 1.2)

Product:	Rotstop
Micro-organism:	*Phlebiopsis gigantea* (Fr.) Jülich
Manufacturer and location of plant:	Verdera Oy Luoteisrinne 2 P.O Box 5 FI-02270 (FI-02271) ESPOO, FINLAND
Contact Point:	Mr. Pekka Seiskari Production Manager Phone: +358 10 217 3720 Mobile phone: +358 50 330 4213 Fax: +358 455 0907 E-mail: pekka.seiskari@verdera.fi

B.1.2.3 Trade name or proposed trade name, and manufacturer's development code number of the preparation if appropriate (Annex IIIB 1.3)

Trade name: Rotstop

B.1.2.4 Detailed quantitative and qualitative information on the composition of the preparation (Annex IIIB 1.4)

Rotstop contains 10% active ingredient (*P.gigantea* spores and mycelium), amorphous silica as a carrier (80 %), lime as pH regulator and some residual moisture.

The viability of the packaged product Rotstop is at least $2x10^6$ cfu/g (usually10^6-10^7 living oidia per 1 gram of the product). The level of contaminating micro organisms is less than 1% of the viable count of the active substance *P. gigantea*. (Safety data sheet, Verdera 2003).

PG Suspension is a suspension concentrate composed of live spores (oidia) and mycelium of *P. gigantea*. The formulation contains: spores (0.5%), water (< 33%), sugar and honey (< 67%), cellulose polymer (0.06%), hexacol supra blue (0.02%) (home page of Forestry Commission).

For further details, see the Confidential information, Annex C.

B.1.2.5 Physical state and nature of preparation (Annex IIIB 1.5)

Wettable powder.

B.1.2.6 Function (Annex IIIB 1.6)

Biofungicide

B.1.3 References relied on

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
Annex II Data and Information					
IIB 1.3	Eriksson, J. Hjortstam, K. Ryvarden, L.	1978	The Corticiaceae of North Europe. Vol. 5, pp. 1181 - 1183. Not GLP. Published.	N	
IIB 1.3	Eriksson, J. Hjortstam, K. Ryvarden, L.	1981	The Corticiaceae of North Europe. Vol. 6, pp. 1181 - 1183. Not GLP. Published.	N	
IIB 1.3	Korhonen, K., Kauppila, P.	1988	The sexuality of *Phlebiopsis gigantea* Karstenia. Vol. 27, pp. 23 – 30. Not GLP. Published	N	
IIB 1.3	Vainio, E. J., Korhonen, K., Hantula, J.	1998	Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. Mycol. Res. Vol.102 (2), pp. 187 – 192. Not GLP. Published.	N	
IIB 1.3	Vainio, E. J., Hantula, J.	2000	Genetic differentiation between European and North American populations of *Phlebiopsis gigantea.* Mycologia. Vol. 92(3), pp 136-146. Not GLP. Published.	N	
IIB 1.3	Parmasto, E., Hallenberg, N	2000	A taxonomic study of phlebioid fungi (Basidiomycota). Nord. J. Bot. Vol. 20 (1), pp 105-118. Not GLP. Published.	N	
IIB 1.3	De Koker, T.H., Nakasone, K.K., Haarhof, J., Burdsall, H.H., Janse, B.J.H.	2003	Phylogenetic relationships of the genus *Phanerochaete* inferred from the internal transcribed spacer region. Mycol. Res. Vol, 107(9), pp. 1032-1040. Not GLP. Published.	N	
IIB 1.3 IIB 1.3.3	Holmer, L. Stenlid, J.	2003	New isolates of *Phlebiopsis gigantea*; methods and results. Report. Swedish University of Agricultural Sciences. 9 pp. Not GLP. Unpublished.	Y	VRA
IIB 1.3 IIB 1.3.3	Webber, J., Thorpe, K.	2003	Potential for biological control of *Heterobasidion annosum* in the UK using Rotstop. In: Laflamme *et al.* (eds.). Root and butt rots of forest trees. Proc. 10th Int. conf. on root and butt rots.Quebec City, Canada, 2001, pp. 221 – 225. Not GLP. Published.	N	FOC

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
IIB 1.3	Grillo, R., Hantula, J., Korhonen, K.	2005	Interfertility between North American and European strains of *Phlebiopsis gigantea*. For. Path. Vol. 35, pp. 173-182. Not GLP. Published.	N	
IIB 1.3.1	Anonymous	1993	ATCC Accession form no. 90304 Not GLP. Unpublished	Y	VRA
IIB 1.3.1	Anonymous	2004	DSMZ Accession form no. 16201 Not GLP. Unpublished	Y	VRA
IIB 1.3.1	Anonymous	2004	DSMZ Accession form no. 16202 Not GLP. Unpublished	Y	VRA
IIB 1.3.1	Anonymous	2004	DSMZ Accession form no. 16203 Not GLP. Unpublished	Y	VRA
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD188 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD189 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD190 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD191 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD192 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD193 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD194 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD195 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD196 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2003	IMI Safe deposit certificate SD197 Not GLP. Unpublished	Y	FOC
IIB 1.3.1	Anonymous	2004	Identification of fungus cultures. DSMZ		
IIB 1.3.3	Hallaksela, A-M., Korhonen, K.	1992	Identification of the fungus from the biopreparate made by Kemira Oy for conifer stump treatment. Report. Finnish Forest Research Institute. 4 pp. Not GLP. Unpublished.	Y	VRA
IIB 1.3.3	Hoffman, P.	2004	Identification of fungus cultures. Certificate. DMSZ. Not GLP. Published	N	
IIB 1.3.3	Vainio, E. Lipponen, K. Hantula, J.	2001	Persistence of a biological strain of <u>*Phlebiopsis gigantea*</u> in conifer stumps and its effects on within-species genetic diversity. For. Path. Vol. 31, pp. 285-295. Not GLP. Published.	N	

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
IIB 1.3.3	Korhonen, K.	2003a	Identification of fungal isolates from the biopreparates 1984, 1985 and 1986, made by Verdera Oy for treating conifer stumps against *Heterobasidion*. Report. Finnish Forest Research Institute. 2 pp. Not GLP. Unpublished.	Y	VRA
IIB 1.3.3	Thorpe, K.	2005a	SOP: Method for selection of isolates for PG Suspension. Forest Research, UK. Not. GLP. Unpublished.	Y	FOC
Annex III Data and Information					
IIIB 1.4	Anonymous	2003	Rotstop safety data sheet	Y	VRA

**: Protection for 5 years claimed from date of decision concerning listing in Annex I - the study report has not been submitted any of the Member States in support of an application for authorization, or (though the study report has been submitted) has not been used any of the Member States as the basis for decision on the initial authorization, or to maintain a given authorization, of a plant protection product before the date of submission of the dossier to Rapporteur Member State.*

***: Owners' code identifications and names:* VRA – Verdera; FOC – Forestry Commission

Draft Assessment Report

Phlebiopsis gigantea

Volume 3

Annex B.2

Biological, physical, chemical and technical properties

Rapporteur Member State: Estonia

April 2007

TAIMETOODANGU INSPEKTSIOON

Volume 1

Level 1: Statement of subject matter and purpose for which the monograph was prepared

Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

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Level 3: Proposed decision with respect to the application for inclusion of the active substance in Annex I

Level 4: Further information to permit a decision to be made, or to support a review of the conditions and restrictions associated with the proposed inclusion in Annex 1

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B.2 Biological, physical, chemical and technical properties

B.2.1 Biological properties of the micro-organism (IIB 2)

B.2.1.1 History of the micro-organism and its uses. Natural occurrence and geographical distribution (IIB 2.1)

B.2.1.1.1 Historical background (IIB 2.1.1)

There is a long history of *P. gigantea* use in Europe (Pratt, Niemi & Sierota 2000, Gibbs, Greig & Pratt, 2002, Thor 2003), beginning in the UK with early studies conducted in the 1950's and 1960's by Rishbeth (1963), Greig (1976) and Webb (1973) amongst others. Originally around 62 000ha of pine forest was treated in the UK (Webb 1973), but this has since declined due to problems with adequate supervision of labour, and now only pine plantations in Thetford Forest in Eastern England receive biological treatment.

Biological treatment was brought into Polish forests in the 1970's (Sierota, 2001) in the form of PG IBL. This product utilises local isolates of *P. gigantea* and consists of beech sawdust and *P. gigantea* spores and mycelium. It is officially recommended for first pine thinnings.

Stump treatment research in Finland in the 1970's (Kallio & Hallaksela, 1979) led the agrochemical company Kemira Agro Oy to supply foresters with agar plates colonised with *P. gigantea*, from which the spores were washed and applied to pine stumps in aqueous solution. Sales were not sufficient to maintain production however. In 1991 a strain of *P. gigantea* isolated from a *Picea abies* stump was formulated into a dry powder and used in field trials in Finland, Sweden and Norway (Korhonen *et al*. 1994) where it was found to efficiently exclude *H. annosum* from stumps also in mechanised stump treatment (Thor & Stenlid, 1998). This Finnish isolate of *P. gigantea* was commercialised under the trade name Rotstop, which is currently manufactured and marketed by Verdera Oy (formerly Kemira Agro Oy) in Fennoscandia. In addition, a parallel product Rotstop S, based on a Swedish isolate of *P. gigantea*, was recently developed and is registered for use in Sweden.

B.2.1.1.2 Origin and natural occurrence (IIB 2.1.2)

P. gigantea is found throughout the temperate Northern Hemisphere and has also been recorded in southern Europe, East Africa, Central America, Australia and New Zealand. There is a relatively high degree of variation within populations of *P. gigantea*. However, studies of sexual compatibility indicate that all European populations are interfertile, demonstrating that *P. gigantea* is a single species and there are no distinct geographic eco-types. This is further supported by the considerable levels of interfertility recently found between European and North American (including US and Canadian) isolates (Korhonen & Kauppila 1988; Vainio *et al*. 1998; Vainio & Hantula 2000; Hollmer & Stenlid 2003; Webber & Thorpe 2003; Grillo *et al.* 2005). This DAR covers a number of different isolates of *P. gigantea* found in Fennoscandia and the UK.

P. gigantea is a saprophytic wood-rotting fungus, causing a typical white rot of coniferous timber. The fungus is a primary coloniser of wood, and requires high moisture content for its growth. (Typically it colonises freshly-cut stems with moisture contents in excess of 150% of dry weight). It is one of the commonest decayers of pine logs in the UK, and can cause significant degrading of produce left for too long in the forest. Freshly cut stumps are often naturally colonised by *P. gigantea* regardless of the application of any stump treatment agent. The fungus can survive in stumps at temperatures of more than 30^{o}C and well below freezing respectively, and competes with *Heterobasidion annosum* under such conditions.

Fruit bodies are common in woodland and grow on decayed wood in contact with the ground, for example on the underside of fallen trees and branches, on stumps, and on the ends of stacked logs. They are inconspicuous, irregular, resupinate short-lived structures. Colourless basidiospores 5-8 x 2.5-4 µm are liberated from ripe sporophores. The spores are widely dispersed by air, and have been trapped 250 miles from the nearest likely source. More spores are liberated at night than during the day. The rate of sporulation is reduced by extremes of temperature, and is inhibited during periods of hot, dry weather, or when sporophores are frozen. In temperate climates such as Britain sporulation occurs in all months of the year, and in any one location it may be effectively continuous, albeit from a number of changing sources. In Britain, spores have been trapped throughout the country, even as far from any potential source as Scalloway, Shetland Islands.

P. gigantea also produces asexual spores called oidia, which arise from the segmentation of hyphae. This form of the fungus is used in the MPCP. The role of oidia in the life cycle of *P. gigantea* in the wild is not fully understood, in that although such spores are produced naturally and are capable of infecting timber, their viability and mode of spread has not been studied (Käärik & Rennerfelt 1957; Meredith 1959; Rishbeth 1959; Meredith 1960; Gremmen 1963; Boyce 1966; Kallio 1970; Hallaksela 1977; Petäisto 1978; Holdenrieder & Greig 1998; Pratt et al. 1999; Roy et al. 2003; Grillo et al. 2005).

B.2.1.2 Information on target organism(s) (IIB 2.2)

B.2.1.2.1 Description of target organism(s)

Reference: Korhonen, K., Stenlid, J. (1998): Biology of *Heterobasidion annosum*. In: Woodward *et al*. (eds). *Heterobasidion annosum*. Biology, Ecology, Impact and Control. CAB International, UK, pp. 43 – 70.

Not GLP. Published.

Results: *P. gigantea* is used to control root and butt rot in conifers caused by the European intersterility groups (P, S and F) of the *Heterobasidion annosum* complex, formerly *Fomes annosus*, but now often described in terms of *H. annosum sensu stricto* (Fr.) Bref. (host species *Pinus* and many other species), *H. parviporum* Niemelä & Korhonen (host species mostly *Picea abies*) and *H. abietinum* Niemelä & Korhonen (host species *Abies*). *H. annosum* is widely distributed in the Northern Hemisphere and, in addition to the European complex, American P and S groups have also been identified, the former widely distributed across North America and the latter restricted to the west. Partial sexual compatibility exists between many members of the complex, including the American group, although in nature hybrids appear to be rare. For simplicity the following text will use *H. annosum* to cover the 3 European sub-species.

The fungus gains entry into conifer plantations by infecting the surfaces of freshly-cut stumps with air-borne basidiospores which are liberated throughout the year from perennial fruit bodies and are capable of travelling long distances (300 miles). Infection of a stump surface is followed by rapid growth into the stump mass, down to the smaller roots. This saprophytic phase in the life cycle of the fungus is of no economic importance *per se*. However, although *H. annosum* is unable to live freely in the soil, it can spread from an infected stump root into the root system of a healthy tree where roots are in contact. A pathogenic phase ensues, in which healthy roots are killed by the pathogen and the affected tree may die or, in the case of many conifer species, may suffer from heart-rot. *H. annosum* can remain alive in stumps for many years, and survive from one crop of trees into the next. It thus has the capability of increasing in intensity on a site to the extent that industrial forestry can become uneconomic.

H. annosum has been recorded on more than 200 species of woody plants but is particularly significant in coniferous forests including (amongst others) *Picea abies* and *P. sitchensis*, *Pinus sylvestris*, *P. cembra*, *P. nigra*, *P. pinaster*, *P. pinea* and *P. peuce* and *Abies alba, A. cephalonica*, *A. borisii-regis* (Southern Europe) and *Abies sibirica* in Russia. Damage depends on a number of site factors, one of the most significant being the prevailing soil conditions, with higher risks of infection on well drained soils, and soils of high fertility and lime content in the rooting zone. Infection is also higher on arable/pasture land than heathland or old forest soils (depending on previous management practices). The losses in revenue from direct mortality, decay, reduced tree increment, and reductions in pulping properties result in *H. annosum* being one of the most severe long-term threats to plantation conifers world-wide.

B.2.1.2.2 Mode of action (IIB 2.2.2)

P. gigantea is able to prevent colonisation of stumps by *H. annosum* through competition for resources. Although there may be a degree of hyphal interference of *H. annosum* by *P. gigantea*, there is no evidence in the available literature that *P. gigantea* controls *H. annosum* by antibiotic or toxic means.

B.2.1.3 Host specificity range and effects on species other than the target harmful organism (IIB 2.3)

P. gigantea is a saprophytic wood-colonising decay fungus, which is used for the control of members of the *Heterobasidion annosum* complex, forest pathogens causing decay and death in living coniferous trees. In fresh

stumps, the main infection entrance route for the pathogen into a tree stand, these fungal species occupy the same ecological niche, i.e. compete for living space and nutrient resources. In stump treatment a high amount of spores of *P. gigantea* is applied on the freshly cut stump surfaces, thereby preventing the establishment of air-borne infection by *Heterobasidion* spp.

The effect of stump treatment with *P. gigantea* on other wood-colonising fungi in stumps has been investigated in several studies. According to these studies, stump treatment with *P. gigantea* is not likely to pose any immediate threat to the genetic diversity of indigenous *P. gigantea* or other fungal populations in coniferous stumps. (Käärik & Rennerfeldt 1957; Thor et al. 1997b; Roy *et al.* 2003; Vainio *et al*. 2001; Vainio *et al.* 2005; Varese *et al.* 2003; Vasiliauskas *et al.* 2004; Vasiliauskas *et al.* 2005).

P. gigantea does not produce any metabolic products toxic to human or animals. It is a non-pathogenic fungus, it is not thermophilic and has an optimal growth temperature of 28°C and a maximum of 38°C, and is not capable of colonising or invading humans or animals, as verified by animal tests. Further animal testing with Rotstop, the representative product based on *P. gigantea*, showed no irritation of skin, eyes or respiratory organs, and there are no records of adverse effects on operators and personnel handling *P. gigantea*-based products, i.e. no irritation or allergenic reactions that would be attributed to the fungal spores in the products (Worgan 1968; Jennison *et al*. 1957; Briggs *et al*. 1975; Hütterman 1997; Holdenrieder & Greig 1998; Ikediugwu *et al.* 1970; Capretti & Mugnai 1989).

Year	Source	Summary of statement
1992	Forestry Commission, UK	It was stated that during development and use of a *P. gigantea* product over the preceding 20 years, no reports had been received of any allergic reactions by users wearing the gloves and protective clothing required for chainsaw operators.
1994	Kemira Agro Oy, Finland	Indicated that there was no evidence of allergic reactions in personnel during research and development and manufacturing of Rotstop. *P. gigantea* has been on the market in Finland since 1978 and between then and the time of writing, no indications of sensitisation were noted in personnel working with the original formulation or a revised formulation launched in 1993.
1995	Forestry Commission, UK	A review of employment records of 21 out of 75 chainsaw operators working between 1945 and 1995 revealed no evidence of occupational health referrals relating to chest complaints or incidences of sick absence associated with the use of *P. gigantea*.
1996	Forestry Commission, UK	All UK PG Suspension used before 1997 was manufactured and handled by the same individual for 25 years, using a consistent method to harvest oidia. During this time, the subject was exposed (usually unprotected) to oidia on *circa* 4,000 agar plates. Although he was sensitive to a number of allergens (dust, pollen etc.), he showed no evidence of an allergic reaction to *P. gigantea* oidia. During the UK evaluation of PG Suspension this man volunteered for a skin allergy patch test using a suspension of *P. gigantea*. The result of the test was uniformly negative.

1997	Kuopio Regional Occupational Health Institute (KATTL), Finland	In an area where Rotstop was extensively used, exposure and health effects were monitored in harvest machine operators, forestry students and their teacher. Exposure was investigated by measuring concentrations of the microorganism in the breathing zone, in wipe samples taken from the cab and by measuring antibodies in blood. Symptoms questionnaires were used to assess health effects. The students also kept a symptoms diary and measured their pulmonary peak expiry flow (PEF) three times per day. No *P. gigantea* spores were found in samples taken from cab air or in wipe samples and there was no rise in antibodies in blood. A range of symptoms, including skin and respiratory effects, were reported but none of the operators (who had been working in forestry for 6-27 years) associated symptoms with the use of Rotstop. Amongst students, only two applied the fungicide and they experienced the most symptoms including variation in PEF. Occupational hygiene and antibody measurements show that there was no exposure to *P. gigantea* spores and, although in students exposure to Rotstop may have been associated with a higher occurrence of respiratory symptoms, this was not the case with the harvest machine operators. Due to the low numbers of subjects involved it is not possible to conclude whether the normal uses of Rotstop have any adverse health effects.
2000	Kemira Oy, Finland	The chief medical officer stated that no clinically significant findings were found in workers associated with the occupational exposure of Rotstop (*Phlebiopsis gigantea*). Workers were subjected to regular examinations including respiratory function tests.
2000	Finnish Forest Research Institute (METLA)	States that during use of *Phlebiopsis gigantea* for the past 20 years, in field and laboratory conditions, there have been no allergic reactions or sensitisation reported by personnel, who were surveyed.
2002	Kuopio Regional Occupational Health Institute (KATTL), Finland	The study compared the incidence of dermal and respiratory symptoms in harvester operators exposed to a range of biological fungicides, log marking pigments and oils with the incidence in a control group consisting of timber truck drivers and forestry technicians. Exposure was also measured using air samples taken from the breathing zone and harvester cab filters and compared with samples taken from outdoor air. Harvester operators had higher rates of asthma and other respiratory symptoms compared with control subjects even though concentrations of viable spores in the breathing zone and outdoor air were comparable. Incorrect mixing of fungicide spray (Rotstop) can expose the operator to an irritating dust and there was some correlation between incorrect mixing and the incidence of respiratory symptoms and asthma. The correlation was not statistically significant and these subjects also smoked more frequently than others. Mild skin conditions (eczema and dermatitis) were also higher in the operators compared with controls and correlated with both handling hydraulic hoses and the use of the biological fungicide. When used correctly there should be almost zero skin exposure, but skin contamination may occur during maintenance
2004	Kuopio Regional Occupational Health Institute (KATTL), Finland	Respiratory exposure to *P. gigantea*, other microorganisms and endotoxins was assessed using air samples from the workers breathing zone. Dermal exposure was assessed using a fluorescent tracer method. Systemic exposure was also investigated by antibody determinations in serum samples. Respiratory and dermal exposure to *P. gigantea*, log colour-coding agents, hydraulic and chainsaw oils was insignificant during normal harvester operations; exposure may occur during maintenance work but can be controlled by the use of appropriate personal protective equipment.

2005	Forest Research, UK	A survey was conducted of 7 forestry workers who used PG Suspension and 2 personnel involved in manufacture of the product. Four of those surveyed had been using the product daily for more than 5 years. Four of these had some hand/arm contact with the formulation but had not experienced any adverse effects. Two others who had asthma or eczema had also not experienced any adverse effects. Only one of the workers, who was exposed less than once per week, recorded any effects (skin dermatitis) but considered that this was unrelated to working with PG Suspension.
2005	Verdera Oy, Finland	Based on medical examination and clinical follow-up studies of all workers who may have been exposed to Rotstop (*Phlebiopsis gigantea*) dust, a specialist in occupational health care states that there were no significant findings related to occupational exposure. No one had acute or chronic symptoms, and there were no details on occurrence of hypersensitivity or chronic sensitisation.

B.2.1.4 Development stages/life cycle of the micro-organism (Annex IIB 2.4)

P. gigantea is a common primary coloniser of dead conifer wood in boreal and temperate forests throughout the world. The fungus causes a typical white rot of coniferous timber, especially pine. Fruit bodies are formed frequently on decayed wood in contact with the ground, for example on the underside of fallen trees and branches, on stumps, and on the ends of stacked logs. Fructification occurs normally within one year after infection and continues for up to three or even four years. The fruit bodies are often large, irregular, resupinate structures. Sexual basidiospores are liberated from the ripe sporophores and are born aloft on air currents. Spore infection is very effective, and usually several individuals of *P. gigantea* can be found on a stump one year after tree felling. In this ecological niche it competes with other wood-rotting fungi, e.g. *Heterobasidion annosum*, *Stereum sanguinolentum* and *Sistotrema brinkmannii*.

The mating system of *P. gigantea* is bipolar, and clamp connections are found on both hetero- and homokaryotic mycelia. Homokaryotic fruiting occurs *in vitro*, but fruit bodies from the field have been shown to be heterokaryotic. Homokaryotic basidiospores from a heterokaryotic fruit body germinate to form genetically different homokaryons. When basidiospores infect a stump, mating between them may occur, producing genetically different heterokaryons. These mycelia do not fuse but compete with each other.

P. gigantea also produces asexual spores called oidia, which arise from the segmentation of hyphae in the aerial mycelium. Both homokaryotic and heterokaryotic isolates of *P. gigantea* form oidia, which is the form of the fungus in MPCP's. When oidia from a heterokaryotic mycelium are applied on a fresh stump surface, the suspension contains both hetero- and homokaryotic propagules. In the stump, the heterokaryotic oidia germinate to form heterokaryons which are identical with the original mycelium. Homokaryotic oidia germinate to form two types of homokaryons, which are compatible and occasionally mate to produce a heterokaryon that is identical with the original one. When the genetically identical heterokaryons meet, they fuse into one mycelial unit. The result is a genetically homogenous heterokaryotic mycelium in the stump.

The role of oidia in the life cycle of *P. gigantea* in the wild is not fully understood, in that although such spores are produced naturally and are capable of infecting timber, little is known about their viability and mode of spread. However, arthropods have been observed among oidia of *P. gigantea* under the bark of stumps and the fungus has been isolated from various arthropods, suggesting that they play a role in the dispersal of the fungus (Korhonen & Kauppila 1988; Holdenrieder & Greig 1998).

B.2.1.5 Infectiveness, dispersal and colonisation ability (Annex IIB 2.5)

Influence of temperature and humidity - The behaviour of *P. gigantea* in the field is strongly determined by the local environment, in particular humidity and temperature.

In the UK Meredith (1959) and Rishbeth (1959) found *P. gigantea* fruit bodies released spores at temperatures ranging from 0-22°C but ceased when the fruit bodies froze. However, freezing (at least down to –5°C) does not seem to affect the ability of fruit bodies to resume spore discharge once defrosted. Desiccation of the fruit body also significantly reduces spore discharge. Spore numbers drop during periods of dry weather as demonstrated in loblolly pine plantations in the south eastern United States (Boyce, 1966), in Scots and Corsican pine in the UK (Meredith 1959; Rishbeth 1959, 1963) and in Scots pine plantations in the Netherlands (Gremmen 1963).

However, fruit bodies can recover in subsequent periods of wet weather. Such sensitivity of fruit bodies to humidity probably accounts for the degree of seasonal variation in stump colonisation found by Meredith. High (80-100%) natural stump infection occurred from late summer through to the spring, followed by a fall in the period April to July (27-56%) when the weather tends to be warm but dry. In contrast, in Finnish studies (Kallio & Hallaksela 1979; Kallio 1970) spores are most abundant in April-May when mean temperatures are between 5 and 15°C, and airborne dissemination declines sharply from mid-September when temperatures fall. In that study precipitation was not found to have any significant impact on spore deposition.

Temperature and humidity also affects the growth rates of colonising *P. gigantea*. The moisture content of the wood within the stump affects growth of *P. gigantea* whereby growth rates are slower in wood with lower moisture content (Meredith 1960). Field reports also indicate *P. gigantea* grows more slowly in the cold winter months than in the summer (Rishbeth 1963).

In vitro studies also demonstrate the influence of temperature on *P. gigantea* growth. At 10°C on malt agar Cartwright and Findlay (1958) reported daily increments of 6mm and Thorpe (2001) reported 5.4mm. This increased to 16mm at 27.5-28°C. Thorpe reported a cessation of growth of PG 410.3 (and other isolates from within the 14 supported in this dossier) at 35°C, although this temperature did not prove to be a lethal limit as samples re-incubated at lower temperatures recovered. Cartwright & Findlay (1958) report *P. gigantea* is killed at temperatures exceeding 38°C. Niemi (1992a) compared the growth rate on pine and spruce sawdust agar of VRA 1835 with the growth rates of *H. annosum* P and S-type strains at temperatures ranging from 4°C to 28°C. Growth of both fungi was negligible at +4°C. VRA 1835 grew equally well on both substrates at temperatures of 8°C and higher, with the highest daily increment of 8 mm/day at 28°C. The *H. annosum* strains showed optimum growth (5-6 mm/day) at 22°C. The effect of high temperature on the viability of *P. gigantea* spores was assessed by keeping a working solution of Rotstop for 10 minutes at temperatures between 30°C and 80°C (Niemi 1992b). Spore counts somewhat decreased at 30- 45°C, and all spores were killed at 50°C and above. Thor *et al*. (1997a) also observed decreased spore viability during 8 hours at 35°C and higher temperatures.

<u>Survival during practical stump treatment</u> - *P. gigantea* spores can be applied to stumps manually but these days are more often applied through harvesting machinery. This involves the spore solutions being pumped from a tank on the back of the harvester along pipe work leading down to the cutting blade. The solution is either sprayed onto the stump and bar as it cuts through the timber, or it is sprayed through holes drilled directly into the cutting bar. During transit from the tank to the stump surface spores can undergo conditions of increased pressure and temperature. Laboratory and field-based studies have therefore been conducted in the UK (Burchby, 2001) and Sweden (Thor *et al*., 1997a) to ensure that such conditions do not have unacceptable impacts on spore viability. Results indicate that spores can survive high temperatures (up to 60°C) for short periods of time, and elevated pressures of 1600-2200 kPa, and that viability remains well within acceptable levels during mechanical application.

<u>Nutritional requirements</u> – *P. gigantea* is highly adapted to the selective medium presented by freshly cut stumps where it, along with *H. annosum*, is one of very few fungal organisms capable of growth in the initial stages after felling (Meredith, 1960; Rishbeth, 1963). *P. gigantea* will grow on many conifer species including pine, spruce, larch and Douglas fir, although growth rates on the latter species tends to be lower (Thomsen & Jacobsen, 2001; Rishbeth, 1963). Different isolates of *P. gigantea* vary slightly in growth rates, but generally speaking within pine species it appears *P. gigantea* is slower to colonise Corsican pine than Scots pine (Rishbeth, 1963), and lower growth rates are seen in Sitka as compared with Norway spruce (Thomsen & Jacobsen, 2001). In addition to conifer species the fungus can also be grown on sunflower husks (Demchenko, 1999), chips of poplar veneer and beech sawdust. The latter has been exploited as the growth medium in the Polish bio-control product PGIBL.

Although wood provides an acceptable medium for growth, spores do not germinate on other parts of the tree. They are readily collected from foliage in woodland containing viable fruit bodies, but cannot germinate on such a medium and viability drops rapidly over a period of 0-10 days (Rishbeth 1959). *In vitro* studies show *P. gigantea* readily grows on general carbon-based laboratory media such as malt agar, malt extract agar and malt agar enriched with Phostrogen, a plant fertiliser high in nitrogen and potash.

<u>Light requirements</u> – *P. gigantea* is capable of growth both in the light and dark. However, Kallio reports that spore release from fruit bodies is higher in the field at night than during the day. Demchenko (1999) found no differences in biomass accumulation of mycelium in varying light regimes, but found the rate of sporulation of cultures was higher with alternating dark-light periods compared with cultures grown in the dark.

References used to generate the above summary follow in geographical order:

<u>Scandinavia</u>

Kallio, T (1970): Aerial distribution of the root-rot fungus *Fomes annosus* (Fr.) Cooke in Finland. Acta Forest. Fenn. Vol 107, 55 pp.

Not GLP. Published.

Results: Spore deposition rates of *H. annosum*, *P. gigantea* and *Trichoderma viridae* in Finland were measured with the aid of spruce discs and agar plates. Sites were situated from Helsinki to as far north as Ivalo. Spore deposition of *P. gigantea* almost coincided with that of *H. annosum*, occurring from April to late October, peaking in July. Highest deposition occurred at night.

Kallio, T., Hallaksela, A-M. (1979): Biological control of *Heterobasidion annosum* (Fr.) Bref. (*Fomus annosus*) in Finland. Eur. J. For. Path. 9(5), pp. 298-308.
Not GLP. Published.

Results: In this study in Finland the efficacy of 5 different fungi (*P. gigantea*, *Botrytis cinerea*, *Gliocladium deliquescens*, *Trichoderma viridae* and *Verticicladiella procera*) was tested against *H. annosum* on Norway spruce stumps. The potential antagonists were inoculated onto stumps in thinnings carried out monthly for a year. *P. gigantea* completely excluded *H. annosum* and was also found to have colonised 33% of untreated stumps, although airborne dissemination of this fungus, and consequent colonisation decreased after mid-September and throughout the winter.

Niemi, M (1992a) Effect of temperature on the growth of *Peniophora gigantea* and *Heterobasidion annosum*. Kemira Agro Oy, Espoo Research Centre. Test report 9241, 2 pp.

Not GLP. Unpublished.

Results: In this experiment one strain of *P. gigantea* (VRA 1835, the Rotstop isolate), *H. annosum* (P-type) and *H. annosum* (S-type) respectively, were grown on agar plates with pine or spruce sawdust as the sole carbon source. The plates were incubated at temperatures ranging from 4°C to 28°C, and the daily mycelial growth rate was measured. Growth of all strains was negligible at 4°C. From 8°C upwards the growth rate of *P. gigantea* increased with increasing temperature, reaching a maximum of 8 mm/day at 28°C. Both strains of *H. annosum* had optimum growth (5-6 mm/day) at 22°C. The three strains grew equally well on both pine and spruce substrate.

Niemi, M (1992b): Effect of high temperature on the viability of the spores of *Phlebiopsis gigantea.* Test report 9252, 1 p.

Not GLP. Unpublished.

Results: The effect of high temperature on the viability of *P. gigantea* spores was assessed by keeping a working solution of Rotstop, containing 1.6×10^7cfu/L, for 10 minutes at temperatures between 30°C and 80°C and thereafter assessing the viability by plate counting. Spore counts were somewhat reduced at 30, 35 and 40°C. At 45°C the temperature effect was more pronounced and the spore count had dropped to 5.9×10^5 cfu/L. All spores were killed at 50°C and higher temperatures.

Thomsen I., Jacobsen, J.B. (2003) Testing of Rotstop on Sitka spruce, Douglas-fir and larch. In: Laflamme *et al*.(eds.). Root and butt rots of forest trees. Proc.10th Int. Conf. on Root and Butt Rots. Quebec City, Canada, 2001, pp. 216-220.

Not GLP. Published.

Results: In Denmark *P. gigantea*, in the form of Rotstop, was used to protect stem discs of larch, Douglas fir, Norway spruce and Scots pine from artificially inoculated *H. annosum* originally isolated from Douglas fir. Mycelial cover of both control agent and pathogen were measured to determine the efficacy of treatment. Rotstop prevented colonisation by *H. annosum* but marked differences in the growth of the mycelium on the 4 species was noted, with most rapid growth evident on larch and Norway spruce and greater colonisation of sapwood than heartwood.

Thor, M, Bendz-Hellgren, M., Stenlid, J. (1997a) Sensitivity of root rot antagonist *Phlebiopsis gigantea* spores to high temperature or pressure. Scand. J. For. Res. 12, 356-361.

Not GLP. Published.

Results: The sensitivity of *P. gigantea* spores to high temperature and pressure was studied by setting an aqueous spore suspension under pressure, or keeping the suspension at temperatures of 20, 30, 35 and 40°C for up to 72 hours. Pressure of 2200k Pa did not affect spore viability. At 20°C the spore count remained unchanged during 8 hours, and the slight decrease observed after 72 hours was not significant. Spore germination had an optimum at 30°C and reached a maximum after 8 hours, but at higher temperatures it decreased over time. Mechanised stump treatment in the field, where spores are transiently exposed to higher temperatures and pressure, did not affect the spore viability.

The Netherlands

Gremmen, J. (1963): Biological control of the root-rot fungus *Fomes annosus* (Fr.) Cke by *Peniophora gigantea* (Fr.) Masse. Med Barbouw. Ned. Bosb. Tijdschr. Vol. 35(9), pp. 356-367.

Not GLP. Published.

Results: In field experiments Gremmen observed that whilst *P. gigantea* would colonise stumps and branches of pine species in the Netherlands, it would not colonise Douglas fir or Japanese larch. Colonisation on pine was dependent on spore deposition, which was depressed during dry periods.

The UK

Burchby, J. (2001): The effect of mechanical application on the viability of *Phlebiopsis gigantea* for the control of *Heterobasidion annosum* root rot of *Pinus* species.
Dissertation. University of Wales. 43 pp.

Not GLP. Unpublished.

Results: Laboratory trials were set up to investigate the effects of elevated temperatures and pressures on *P. gigantea* spore viability. They indicated that spores suffered no loss in viability at 30°C over 6 hours exposure. At 40°C viability began to decrease after 10 minutes, and at 50 and 60°C there was complete loss of viability after 2 minutes and 1 minutes respectively. Furthermore, field trials compared the viability of spore solutions made up in controlled conditions with solutions which had been passed through harvesting machinery (Timberjack 1270B) used in East Anglian forests in the UK. There was no unacceptable decline in spore viability during mechanical application.

Cartwright, K,ST.G., Findlay, W.P.K. (1958): Decay of timber and its prevention.
Forest Products Research Laboratory. 2nd Edition. Her Majesty's Stationery Office, London, pp.178-179.

Not GLP. Published.

Results: In their book 'The Decay of Timber and its Prevention' Cartwright and Findlay provide growth rates of *P. gigantea* on malt agar (see above in main text) and discuss its role as a causal agent of decay in softwood. Although *P. gigantea* can cause decay of timber left lying in the forest, once the wood is seasoned and dried *P. gigantea* is no longer problematic as a high moisture content is needed for growth.

Meredith, D.S. (1959): The infection of pine stumps by *Fomes annosus* and other fungi.
Ann. Bot. Lond. (n.s), Vol. 23 (91) pp. 455 – 476.

Not GLP. Published.

Results: Meredith investigated the infection biology of stump-colonizing fungi in managed pine forests in Eastern England. He showed that *P. gigantea* colonisation of stumps was dependent on spore availability and infections peaked in the winter and early spring. If fruit bodies dried out they became horny and parchment like and ceased spore discharge. But following wet weather led to the rapid reappearance of actively discharging fruit bodies. *P. gigantea* fruit bodies released spores at temperatures ranging from 0-22°C but ceased when the fruit bodies froze. However, freezing (at least down to –5°C) for 10 days did not adversely affect them, and spore discharge was resumed a few hours after thawing

Meredith, D.S. (1960): Further observations on fungi inhabiting pine stumps.
Ann. Bot. Lond.(n.s), Vol. 24 (93), pp. 63-78.

Not GLP. Published.

Results: Meredith compared rates of growth of *P. gigantea* and other fungi (including *H. annosum*) in pine stumps and on artificial media. Low temperatures and low moisture content of stumps reduced growth rates.

Rishbeth, J. (1959): Dispersal of *Fomes annosus* Fr and *Peniophora gigantea* (Fr.) Massee. Trans. Brit. mycol. Soc.Vol. 42 (2), pp. 243 – 260.

Not GLP. Published.

Results: Dispersal and deposition of *P. gigantea* and *H. annosum* spores was measured by exposing freshly cut pine discs or sterilised muslin sheets in an East Anglian forest in the UK. Mean spore deposition levels of 5-23 viable spores $100cm^{-2}$ hr^{-1} were recorded over a year (1957-58). Deposition was highest in still air, and in the vicinity of fruit bodies, and was decreased in periods of dry weather. There was no pronounced seasonal variation in spore levels. Spore deposition levels were found to be similar in Swedish forests visited by Rishbeth. Spores were isolated from foliage, although they are not thought to persist or remain viable on this medium for long periods of time.

Rishbeth, J.(1963): Stump protection against *Fomes annosus*. III. Inoculation with *Peniophora gigantea.* Ann. Appl. Biol. Vol. 52 (1), pp. 63 – 77.

Not GLP. Published.

Results: In experiments to examine the ability of *P. gigantea* to prevent colonisation of conifer stumps by *H. annosum*, Rishbeth compared the growth of *P. gigantea* on Scots and Corsican pine. *H. annosum* was successfully controlled by the antagonist in both pine species but colonisation of Corsican pine was slower than Scots. Colonisation of European larch and Douglas fir was much lower, and *P. gigantea* failed to control *H. annosum* adequately.

Thorpe, K. (2001): Linear growth rates of *Phlebiopsis gigantea* on artificial media. Forest Research, Alice Holt Lodge. Report PPP01020, 3 pp.

Not GLP. Unpublished.

Results: Thorpe compared the linear growth rates on malt agar of a selection of isolates of *P. gigantea* from the UK and Scandinavia, at temperatures ranging from 5°C to 35°C. Mean growth at 10 and 27.5°C compared well with those described by Cartwright and Findlay (IIM 2.8/06). No growth was observed in any isolates at 35°C, but all isolates recovered when sub-cultured and then incubated at 25°C.

The Ukraine

Demchenko, S-I. (1999): Optimization of cultivation conditions for *Peniophora gigantea* (Fr) Mass. (Corticiaceae). Ukrainskii-Botanichnii-Zurnal. Vol. 56(2), pp 192-197. Abstract.

Not GLP. Published

Results: *P. gigantea* was grown under different growth substrates and light regimes to optimise cultivation conditions. From a selection of the following; must-agar, wort, sunflower husks, chaff, sunflower seed cake, cereal bran and sawdust (species unspecified), the most suitable substrates for growth appeared to be chaff and sunflower husks. Alternating light/dark regimes optimised sporulation.

The USA

Boyce, J.S. (1966): Sporulation by *Peniophora gigantea* with reference to control of Annosus root rot. For. Sci. Vol. 12 (1), pp. 2-70.

Not GLP. Published.

Results: Boyce describes the incidence of *P. gigantea* fruit bodies on loblolly pine in the eastern United States. Fruit bodies were commonly formed on stumps, bark sections and fallen branches within a year of thinning. Fruit bodies and spore presence (recorded using Petri dish spore traps) were recorded in every month of the year. Spore deposition seemed to be positively related to precipitation, and similar to deposition rates measured by Rishbeth in Eastern England.

B.2.1.6 Relationships to known plant or animal or human pathogens (Annex IIB 2.6)

A database search was made using DIALINDEX (search term 'ALL SCIENCE') to investigate any reports of pathogenicity to humans and other mammals.

Of the genera grouped under Phanerochaetaceae there were no reports of deleterious impacts on human (or other animal) health. Other genera unrelated to *Phlebiopsis* - *Erythricium* and *Corticium* (synonym *Sclerotium*) yielded reports of pathogenicity to plants. In tropical and sub-tropical regions of Asia, Africa and South America *E. salmonicolor* causes pink disease on tree species including *Citrus*, *Eucalyptus* and *Acacia* leading to the development of cankers and die-back. *Sclerotium rolfsii* is a soil-borne fungus which causes 'Southern Blight,' leading to rot and die-back in many vegetable crops in the tropics, subtropics, and other warm temperate regions. It is clear that both occur in ecological niches and climatic conditions very different from those inhabited by *P. gigantea*.

B.2.1.7 Genetic stability and factors affecting it (Annex IIB 2.7)

Action by *P. gigantea* against *H. annosum* operates through direct competition and some hyphal interference (see Point B.2.1.2.2). These traits, which are a combination of characters such as spore germination, growth rate and wood-colonising ability, are all under continuous, stable, polygenic genetic control, and are not controlled by only a few major genes. The advantage of traits under polygenic control is that they are not subject to breakdown or loss of action via mutation, which can be the case for traits controlled by one or a few major genes.

The system of somatic incompatibility which operates in *P. gigantea* (and other fungi), prevents hyphal fusions between individuals, so horizontal transfer of genetic elements via hyphal fusion is an unlikely event.

In relation to stability, the isolate VRA 1835 has been in use for over 10 years with no discernible change in appearance or efficacy (Korhonen 2003b). It is therefore considered to be a genetically stable organism.

To maintain the quality and consistency of the isolates used to formulate the product, serial subculturing is avoided. Master and seed stock cultures, which are used as the starter inoculum for each new batch of product, are preserved at 4°C and -80°C (Seiskari 2005a; Thorpe 2005a).

For further details see document C.

B.2.1.8 Information on the production of metabolites (especially toxins) (Annex IIB 2.8)

P. gigantea is a saprophytic, wood-rotting basidiomycete fungus and is not listed in standard texts as a toxic organism (e.g. Wyllie & Morehouse, 1977-1978). Worgan (1968) lists it as an edible fungus, and Jennison *et al.* (1957) report animal feeding experiments with *P. gigantea* fungal mycelium.

In one study (Briggs *et al*. 1975), investigating metabolites produced by various fungi in liquid culture, Lup-19(22)-ene and Lupa-15,19(22)-diene were found in the neutral fraction of chloroform extract, and 2',3',5'-trimethoxy-p-terphenyl was detected in the neutral fraction of an ethyl acetate extract of the mycelium. According to Hütterman (1997), this compound is a typical secondary fungal metabolite, and substances like this have been found in almost all wood-inhabiting fungi that have been analysed for the presence of this kind of compounds. This particular compound definitely has a lower toxicity than many other secondary metabolites which have been isolated from fungi against which *P. gigantea* is antagonistic. It is not considered to pose any special harm in the following scenarios: (i) acute toxicity during application, (ii) toxicity on the treated stump, (iii) accumulation in the wood and on the forest floor. Lup-19(22)-ene and Lupa-15,19(22)-diene belong to a class of substances which are widely distributed in nature, e.g. in the bark of trees, in leaves and stems of annual plants, or in seeds. No high toxicity can be expected from these compounds in the case of stump treatment.

There are no other records of metabolites produced by *P. gigantea* that would be of concern for human health and/or the environment (Holdenrieder & Greig, 1998). Studies conducted by Ikediugwu *et al.* (1970) and Capretti & Mugnai (1989) indicate that *P. gigantea* does not depend on the production of toxins for its ability to combat *H. annosum*, but acts through competition for the wood resource.

B.2.1.9 Antibiotics and other anti-microbial agents (Annex IIB 2.9)

Pulkkanen, H. (1996): Antibiotic sensitivity of *Phlebiopsis gigantea*.
Kemira Agro Oy, Espoo Research Centre. Test report 186/96. 3 pp.

Not GLP. Unpublished.

Results: A study was made to investigate the sensitivity of *Phlebiopsis gigantea* to typical antibiotics used against dermatophytes.

The sensitivity of *P. gigantea* to amphotericin B, chlotrimazol, nystatin and pimaricin was tested as follows: the tested antibiotics were weighed and suspended in a small volume of sterile water, which was spread on Potato Dextrose Agar (PDA) plates to give concentrations of 0.02, 0.1 and 0.5 mg/ml on the plates.

A suspension of Rotstop, containing *P. gigantea* as the active substance, was diluted to 10^{-4}, 10^{-5} and 10^{-6} and spread on replicate PDA-antibiotic plates of each concentration. As controls were used PDA plates without the antibiotic. The number of colonies of *P. gigantea* on the plates was counted after 3, 7 and 14 days incubation. The effect of the antibiotics on the viability of *P. gigantea* was calculated as cfu's per gram of Rotstop.

P. gigantea was highly sensitive to chlotrimazol and pimaricin at all concentrations, while nystatin inhibited colony formation completely at the two higher concentrations and reduced vialbility at the lowest concentration. The fungus showed the lowest sensitivity to amphotericin B, which completely inhibited fungal growth only at the highest concentration. At the lower concentrations the growth rate of the fungus was reduced, but the number of colonies was not affected.

B.2.2 Physical, chemical and technical properties of the plant protection product (Annex IIIB 2)

B.2.2 Appearance (colour and odour) (Annex IIIB 2.1)

Reference:	Woolley, A.J., Mullee, D.M. (2004a): Determination of accelerated storage stability. SPL Project number: 1841/005. SafePharm Laboratories. 27 pp. Unpublished. Woolley, A.J., Mullee, D.M. (2004b): Determination of long-term storage stability. SPL Project number: 1841/006. SafePharm Laboratories. 27 pp. Unpublished.	
Test Material:	Commercial Rotstop batch	
GLP:	Yes	
Results:	Colour:	Cream powder
	Odour:	Weak fungus like odour
	Physical state:	Cream,opaque fine powder
RMS comments:	The methods and the results are acceptable.	

B.2.2.2 Storage stability and shelf-life (Annex IIIB 2.2)

B.2.2.2.1 Effects of light, temperature and humidity on technical characteristics of the plant protection product (Annex IIIB 2.2.1)

Table B.2.2.2.1a: Accelerated storage

Reference:	Woolley & Mullee (2004a) Woolley & Mullee (2004b)
Test Material:	Commercial Rotstop batch
Method:	The study was conducted according to Methods of the CIPAC Handbook for the Analysis of Technical and Formulated Pesticides Methods of Commission Directive 92/69/EEC and the current OECD Guidelines for testing of Chemicals.
GLP:	Yes
Results:	There was no significant change in the appearance of the formulation or container of the test material during storage at 28 ± 2 ºC for 7 days and 8 ± 2 ºC for 1 year.
RMS comments:	The methods and the results are acceptable.

Table B.2.2.2.1b: Accelerated storage

Reference:	Seiskari, P. (2005j): Storage stability of Rotstop. Report-149-1. Verdera Oy. 2 pp. Not GLP. Unpublished.
Test Material:	Commercial Rotstop batch
Method:	Eight commercial batches of Rotstop were stored in plastic sample holders in a refrigerator at +4 ºC and in incubation chamber at +28 ºC.

Viability of samples was analysed according to SOP-101-1 "Viability determination of biological pesticides".

Results: There was no significant change in the appearance of the formulation or container of the test material during storage at +28 ºC for one month and at +4 ºC at least one year (viability over $2x10^6$ cfu/g).

RMS comments: The methods and the results are acceptable.

The table below (Table B.2.2.2.1c) describes the physical-chemical properties of the representative product Rotstop.

Table B.2.2.2.1c: Chemical and physical properties of Rotstop, initially and after 7 days and 1 year of storage

Test	Results		
	Initial	**7 days at 28 ± 2 ºC Woolley & Mullee (2004a)**	**1 year at 8 ± 2 ºC Woolley & Mullee (2004b)**
Formulation	Cream, opaque fine powder which formed clumps.	Cream, opaque fine powder which formed clumps. No caking of test material observed.	Cream, opaque fine powder which formed clumps. No caking of test material observed.
Odour (subjective assessment)	Weak fungus like odour	Weak fungus like odour	Weak fungus like odour
Container	Silver opaque foil sachet, with a white manufacturers label attached. No signs of corrosion or degradation.	Silver opaque foil sachet, with a white manufacturers label attached. No signs of corrosion or degradation.	Silver opaque foil sachet, with a white manufacturers label attached. No signs of corrosion or degradation.
Weight change	-	<8.47 x 10^{-3}% (loss/gain)	-
pH at 25 ºC: 1% aqueus dispersion	6.29	6.35	6.30
Particle size distribution: **Percentage less than 100 µm** **Percentage less than 10 µm**	 57.9% 11.3%	 57.6% 11.7%	 68.5% 18.1%
Wettability	115 seconds	129 seconds	286 seconds
Persistent foaming (specified field application concentration 0.1% w/v): **Initial** **10 seconds** **1 minute** **3 minutes** **12 minutes**	 A few bubbles produced around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery.	 A few bubbles produced around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery.	 A few bubbles produced around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery.
Suspensibility (specified field application concentration 0.1% w/v)	29.6%	30.0%	22.3%
Wet sieve test	22.1% retained on a 75 µm sieve.	34.0% retained on a 75 µm sieve.	32.9% retained on a 75 µm sieve.

B.2.2.2.2 Other factors affecting stability (Annex IIIB 2.2.2)

Not relevant.

B.2.2.3 Explosivity and oxidising properties (Annex IIIB 2.3)

Not relevant.

B.2.2.4 Flash point and other indications of flammability or spontaneous ignition (Annex IIIB 2.4)

Not relevant.

B.2.2.5 Acidity/alkalinity and if necessary pH value (Annex IIIB 2.5)

Reference: Woolley & Mullee (2004a)
Woolley & Mullee (2004b)

Test Material: Commercial Rotstop batch

Method: The study was conducted according to Methods of the CIPAC Handbook for the Analysis of Technical and Formulated Pesticides Methods of Commission Directive 92/69/EEC (CIPAC MT75) and the current OECD Guidelines for testing of Chemicals.

GLP: Yes

Results: 1% aqueous dispersion: initial pH 6.29, after 7 days pH 6.35, after 1 year pH 6.30

RMS comments: The methods and the results are acceptable.

B.2.2.6 Viscosity and surface tension (Annex IIIB 2.6)

Not relevant.

B.2.2.7 Technical characteristics of the plant protection product (Annex IIIB 2.7)

B.2.2.7.1 Wettability (Annex IIIB 2.7.1)

Reference: Woolley & Mullee (2004a)
Woolley & Mullee (2004b)

Test Material: Commercial Rotstop batch

Method: The study was conducted according to Methods of the CIPAC Handbook for the Analysis of Technical and Formulated Pesticides Methods of Commission Directive 92/69/EEC (CIPAC MT53.3.2) and the current OECD Guidelines for testing of Chemicals.

GLP: Yes

Results: Initial 115 seconds, after 7 days 129 seconds, after 1 year 286 seconds

RMS comments: The methods and the results are acceptable.

B.2.2.7.2 Persistent foaming (Annex IIIB 2.7.2)

Reference: Woolley & Mullee (2004a)
Woolley & Mullee (2004b)

Test Material: Commercial Rotstop batch

Method: The study was conducted according to Methods of the CIPAC Handbook for the Analysis of Technical and Formulated Pesticides Methods of Commission Directive 92/69/EEC (CIPAC MT47.1) and the current OECD Guidelines for testing of Chemicals.

GLP: Yes

Results: Initial: a few bubbles produced around the periphery. After 10 seconds, 1 minute, 3 minutes, 12 minutes: a few bubbles remained around the periphery.

RMS comments: The methods and the results are acceptable.

B.2.2.7.3 Suspensibility and suspension stability (Annex IIIB 2.7.3)

Reference: Woolley & Mullee (2004a)
Woolley & Mullee (2004b)

Test Material: Commercial Rotstop batch

Method: The study was conducted according to Methods of the CIPAC Handbook for the Analysis of Technical and Formulated Pesticides Methods of Commission Directive 92/69/EEC (CIPAC MT15.1) and the current OECD Guidelines for testing of Chemicals.

GLP: Yes

Results: Initial: 29,6%, after 7 days: 30.0%, after 1 year: 22.3%

RMS comments: The methods and the results are acceptable.

Reference: Seiskari, P. (2002): Sedimentation tests with Rotstop preparations made of Swedish *Phlebiopsis* strains. Test report. Verdera Oy. Unpublished.

GLP: No

Test Material: Preparations similar to commercial Rotstop. Preparations made of Swedish *Phlebiopsis gigantea* strains coded 1983, 1984, 1985 and 1986. Also commercial Rotstop.

Method: Each preparation was crushed both manually in a mortar and with a small laboratory sample mill. Commercial Rotstop milled with a hammer mill.
1 g of each preparation was well mixed with 1 liter of water according to the normal instructions for use. This solution was poured into an Imhoff-cone and left standing. The volume of solids sedimented to the bottom was determined after 5, 15 and 30 minutes.

Results: Manually crushed preparations sedimented much faster than milled ones. Commercial Rotstop had clearly the best suspensibility. Large particles sedimented during 30 minutes, but the aqueous solution contained sufficient amounts of *P. gigantea* spores to give good protection against *H. annosum*.

RMS comments: The methods and the results are acceptable.

B.2.2.7.4 Dry sieve test and wet sieve test (Annex IIIB 2.7.4)

Reference: Woolley & Mullee (2004a)
Woolley & Mullee (2004b)

Test Material: Commercial Rotstop batch

Method: The study was conducted according to Methods of the CIPAC Handbook for the Analysis of Technical and Formulated Pesticides Methods of Commission Directive 92/69/EEC (CIPAC MT59.3) and the current OECD Guidelines for testing of Chemicals (OECD 110).

GLP: Yes

Results: Dry sieve: Residue on 100µm sieve: initial 42.1%, after 7 days 42.3%, after 1 year 31,5%
Wet sieve: Residue on 75 µm sieve: initial 22.1%, after 7 days 34.0%, after 1 year 32.9%

RMS comments: The methods and the results are acceptable.

B.2.2.7.5 Particle size distribution (dustable and wettable powders, granules), content of dust/fines (granules), attrition and friability (granules) (Annex IIIB 2.7.5)

Reference: Woolley & Mullee (2004a)
Woolley & Mullee (2004b)

Test Material: Commercial Rotstop batch

Method: The study was conducted according to Methods of the CIPAC Handbook for the Analysis of Technical and Formulated Pesticides Methods of Commission Directive 92/69/EEC and the current OECD Guidelines for testing of Chemicals (OECD 110).

GLP: Yes

Results:

size, µm	initial, %	7 days, µm	1 year, %
< 100	57.9	57.6	68.5
<10	11.3	11.73	18.1
<5	2.2	2.76	5.86
<2.5	1.12	1.37	3.11
<1.25	0.823	0.948	2.00
<6.25	0.677	0.773	1.53

RMS comments: The methods and the results are acceptable.

B.2.2.7.6 Emulsifiability, re-emulsifiablilty and emulsion stability (Annex IIIB 2.7.6)

Not relevant.

B.2.2.7.7 Flowability, pourability (rinsibility)and dustability (Annex IIIB 2.7.7)

Not relevant.

B.2.2.8 Physical, chemical and biological compatibility with other products including plant protection products with which its use is to be authorized (Annex IIIB 2.8)

B.2.2.8.1 Physical compatibility

To avoid the risk of activity reduction in *Phlebiopsis gigantea* biofungicide the product should not be tank mixed with other plant protection products or concentrated fertilizer solutions.

B.2.2.8.2 Chemical compatibility

To avoid the risk of activity reduction in *Phlebiopsis gigantea* biofungicide the product should not be tank mixed with other plant protection products or concentrated fertilizer solutions.

To allow the identification of treated stumps, a water-soluble marker dye tablets are available. Marker tablets are not toxic to *Phlebiopsis gigantea* spores.

B.2.2.8.3 Biological compatibility

To avoid the risk of activity reduction in *Phlebiopsis gigantea* biofungicide the product should not be tank mixed with other microbial plant protection.

B.2.2.9 Adherence and distribution to seeds (Annex IIIB 2.9)

Not relevant.

B.2.2.10 Summary and evaluation of data submitted in B.2.2.1 - B.2.2.9 (Annex IIIB 2.10)

Rotstop is a cream coloured, opaque, fine powder having a particle size of 57,9% less than 100 µm and 11,3% less than 10 µm. The pH of a 1% aqueous dispersion is 6,3. There was no significant change in the appearance of the formulation or container of the product, pH, particle size distribution, persistent foaming or suspensibility during storage at 8 ± 2°C for 12 months. An increase in the wet sieve analysis and wettability was observed.

Rotstop contains 10% active ingredient (*P.gigantea* spores and mycelium), amorphous silica as a carrier, lime as pH regulator and some residual moisture.

The viability of the packaged product Rotstop is at least $1x10^6$ cfu/g. Viable spore counts in the production batches in the range $2x10^6 – 10^7$ cfu's/g dry product, on average $5x10^6$ cfu/g. Technical grade of MPCA is a hypothetical stage in a continuous production process.

For comparison, the alternative formulation PG Suspension (SC) contains a minimum of $3.5x10^6$ cfu/ml suspension concentrate, with an upper limit of $1x10^7$ cfu/ml.

The level of contaminating micro-organisms is less than 1% of the viable count of the active substance *P. gigantea*. Rotstop can be stored for 18 months at -18°C, for one year in a refrigerator at +4°C and one week at room temperature.

Rotstop is a wettable powder which is designed for application as a 0,1% water suspension either through an automatic spraying device installed on the harvesting machine or by manual spraying on conifer stumps. During stump treatment operations the solid carrier will start to sediment without stirring of the working solution at intervals, but the spores of *P. gigantea* will stay in solution.

B.2.3 References relied on

Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N	Owner**
Annex II Data and information					
IIB 2.1.1	Pratt, J.E., Niemi, M., Sierota, Z.H.	2000	Comparison of three products based on *Phlebiopsis gigantea* for the control of *Heterobasidion annosum* in Europe. Biocontrol Science and Technology Vol. 10, pp. 467– 477. Not GLP. Published.	N	
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Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N	Owner**
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IIB 2.1.2	Holmer, L. Stenlid, J.	2003	New isolates of *Phlebiopsis gigantea*; methods and results. Report. Swedish University of Agricultural Sciences. 9 pp. Not GLP. Unpublished.	Y	VRA
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Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N	Owner**
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Annex point/ reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant), Published or not	Data Protection Claimed Y/N	Owner**
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**: Protection for 5 years claimed from date of decision concerning listing in Annex I - the study report has not been submitted in any of the Member States in support of an application for authorization, or (though the study report has been submitted) has not been used in any of the Member States as the basis for decision on the initial authorization, or to maintain a given authorization, of a plant protection product before the date of submission of the dossier to Rapporteur Member State.*

***: Owners' code identifications and names (Code identification: VRA – Verdera; FOC – Forestry Commission)*

Draft Assessment Report

Phlebiopsis gigantea

Volume 3

Annex B.3

Data on application and further information

Rapporteur Member State: Estonia

April 2007

Volume 1

Level 1: Statement of subject matter and purpose for which the monograph was prepared

Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Appendix 3: List of endpoints

Level 3: Proposed decision with respect to the application for inclusion of the active substance in Annex I

Level 4: Further information to permit a decision to be made, or to support a review of the conditions and restrictions associated with the proposed inclusion in Annex 1

Volume 2

Annex A: List of the tests and studies submitted and of information available

Volume 3

Annex B: RMS summary, evaluation and assessment of the data and information

Annex B.1: Identity

Annex B.2: Biological, physical, chemical and technical properties

Annex B.3: Data application and further information.

Annex B.4: Proposals for classification and labelling

Annex B.5: Analytical methods

Annex B.6: Effects on human health

Annex B.7: Residue data

Annex B.8: Fate and behaviour in the environment

Annex B.9: Effects on non-target organisms

Annex B.10: Summary and evaluation of environmental impact

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Volume 4

Annex C: Confidential information and summary and assessment of information relating to the collective submission of dossiers

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B.3 Data on application and further information

B.3.1 Further information on the micro-organism (Annex IIB 3)

B.3.1.1 Function (Annex IIB 3.1)

Biofungicide

B.3.1.2 Field of use envisaged (Annex IIB 3.2)

Forestry

B.3.1.3 Crops or products protected or treated (Annex IIB 3.3)

Currently *P. gigantea* is used for the control of *Heterobasidion annosum* in pine species (mainly Scots pine and Corsican pine) and spruce species (mainly Norway spruce). Spreading of the fungal disease in coniferous forest stands is prevented by treating fresh stumps in thinnings and clear-fellings with a spore suspension of *P. gigantea*. Future use may include other conifer species such as Sitka spruce, Douglas fir, European larch etc, depending on the outcome of on-going and future efficacy trials.

B.3.1.4 Method of production and quality control (Annex IIB 3.4)

Technical Grade of the MPCA is a hypothetical stage in a continuous production process of end-use products with a strain of *P. gigantea* as active substance.

Two methods are used in the production of end-products containing any of the 14 strains of *P. gigantea* which are supported. The two production methods result in different formulations, but both are aseptic, continuous manufacturing processes where cultivation conditions are adjusted for optimal growth of the fungus in order to yield acceptable quality in the end-product. The quality criteria are in principle the same for both formulations.

For further details, see the Confidential information, Annex C.

B.3.1.5 Information on the occurrence or possible occurrence of the development of resistance of the target organism(s) (Annex IIB 3.5)

An overview of the infection and reproductive behaviour of the pathogen is necessary before reviewing the question of resistance.

Primary infection of a healthy stand by the pathogen *Heterobasidion annosum*, *H. parviporum* and *H. abietinum* (henceforth considered as *H. annosum* for clarity) occurs when basidiospores germinate on the surface of a freshly-cut stump, forming a homokaryotic mycelium, the cells of which are multinucleate. These homokaryons are generally weakly virulent, and unable to produce basidiocarps (fruit bodies). Mating with a compatible homokaryon mycelium, results in a heterokaryotic mycelium that is virulent and able to fruit. A compatible nuclear pair fuses in the basidium into a diploid nucleus, and subsequent meiosis produces four haploid nuclei, each of which migrates into a spore. Mature basidiospores are usually binucleate because the nucleus divides once in the spore. Virulent mycelium grows saprotrophically into stump roots, and at points of contact with healthy roots of adjacent standing trees sets up invasive infections which spread from roots into the base of the tree. Infected trees are rendered unstable by the decaying of their roots, and their growth rate is often adversely affected. Basidiospore-bearing sporocarps are produced on the edge of infected xylem in roots or tree buttresses. They are perennial, (up to 20 years) and sporulate profusely (250000 spores/cm^2/hr). Spores are small (4 – 5 µm in diameter), disperse long distances in the air (500 km), and remain viable for several months.

The mode of action of *P. gigantea* against *H. annosum* is thought to rely on direct competition for the woody substrate i.e. no reliance upon the production of specific secondary metabolites (Meredith 1960; Rishbeth 1963; Gremmen 1963; Ikediugwu et al. 1970; Ikediugwu 1976; Capretti & Mugnai 1989; Holdenrieder & Greig 1998). Whichever fungus more rapidly colonises the freshly exposed wood occupies it and retains possession. If *P. gigantea* is applied as a stump treatment rapidly after felling it will retain its hold of the stump, preventing any ingress from *H. annosum*.

No studies are reported in which resistance has been observed, or which have investigated the possibility of the development of resistance within *H. annosum* to applications of *P. gigantea* to stumps. This is perhaps not surprising, since the mode of action is not specific but has a very general basis. Under these circumstances, development of significant resistance to the effects of *P. gigantea* within a population of *H. annosum* seems improbable. Resistance would have to take the form of enhanced tolerance within heterokaryotic *H. annosum* hyphae to competing hyphae of *P. gigantea*. Many genes within the target organism would have to have mutated to neutralise the effect of *P. gigantea*, and it is therefore much less likely that resistance would develop. In addition, heritable tolerance would have to be passed on to a sizeable proportion of the next generation of basidiospores within basidiocarps. To have an economic effect, basidiospores with enhanced tolerance to *P. gigantea* would also need to retain their pathogenicity and out-compete local populations of *H. annosum*, which are subject to incursion by aerial basidiospores from other populations at great distance (at least as far as 45 km). The process from stump infection to sporocarp production may take years within a single stump. At northern latitudes, sporocarps are also long-lived. The process whereby characteristics are inherited in this pathogen, are likely to be very slow.

There are thought to be five intersterility genes that regulate mating within the major groups of the pathogen throughout the world, where six sub-species of the fungus have evolved during the past few thousand years. (In fact, what were previously the sub-species known as the P-type, S and F-type most now consider as separate species - *H. annosum*, *parviporum* and *abietinum*). Although each has its preferred group of host tree species, all are capable of infecting most conifers. This indication of the inability of *H. annosum* to specialize may also demonstrate that the development of resistance to *P. gigantea* (if it ever occurs) is likely to be a very slow process. It is significant to note that *Heterobasidion* and *P. gigantea* have existed together, and competed with each other, for probably thousands of years if not millions, and no signs of resistance have been reported.

In summary, the possible inducement of resistance within the pathogen by stump treatment has received no study. However, although such resistance is theoretically possible, it is thought to be highly unlikely to develop and persist because of the nature of the pathogen life-cycle, and the wide-spectrum mode of action of *P. gigantea.* (K. Korhonen, pers. comm.).

B.3.1.6 Methods to prevent loss of virulence of seed stock of the micro-organism (Annex IIB 3.6)

In order to ensure the consistency and integrity of the master and seed stock, the *P. gigantea* strains are preserved under liquid nitrogen, at –80°C and at 4°C.

Typical features of the strains such as growth rate, growth characteristics of the mycelium and fecundity (oidial production) are continuously monitored in order to reveal any major changes in the behaviour of the strain. Also the ligninolytic enzyme activity of a strain and its competitive behaviour in dual culture with *Heterobasidion annosum* and *H. parviporum* can be assessed at intervals (Hallaksela & Korhonen 1992a; Seiskari 2005c; Korhonen 2003b).

The same standard microbiological methods can be used for all *P. gigantea* strains supported in this dossier. The following procedure is used for the *P. gigantea* strains used in the production of the representative formulation Rotstop:

Reference: Seiskari (2005a). SOP 144-1. Maintenance and subculturing of microbial strains used in Rotstop production. Verdera Oy. 3pp. Not GLP. Unpublished.

Summary:

- seed bank ampoules, containing spores of the strain harvested from malt agar plates, are stored under liquid nitrogen in Verdera's culture collection

- for each strain, a general supply of ampoules is stored in -80°C; a batch of 100 ampoules containing spore suspension is prepared from one seed bank ampoule in the same way as the seed bank ampoules; each batch is checked for viability and visual inspection of growth morphology

- inoculum for production is prepared by plating spore suspension from a general supply ampoule on potato dextrose agar.

Reference: Thorpe (2005a): SOP: Method for selection of isolates for PG Suspension. Forest Research, UK. Not. GLP. Unpublished

Summary: This SOP covers the steps involved in the selection of isolates of the fungus *Phlebiopsis gigantea* for use in the alternative formulation PG Suspension. However, some of the *in vitro* tests outlined are also used to quantify the performance of isolates already in use in PG Suspension. This ensures the consistency and integrity of the master and working seed stock is maintained, as isolates can degrade in culture. *In vitro* growth rates on wood discs and artificial media are performed, in addition to quantification of oidia production on Phostrogen agar.

B.3.1.7 Recommended methods and precautions concerning handling, storage, transport or fire (Annex IIB 3.7)

Technical grade of the MPCA is a hypothetical stage of a continuous production process and no MSDS for the active substance is available. A MSDS for the representative formulation Rotstop is available:

Handling: Avoid contact with skin, eyes and clothing. Do not breathe dust. Wear personal protective clothing, effective dust mask and gloves. Wash hands and face with soap and water before breaks and after handling the product.

Storage: Keep unopened in a dry and cool place at temperatures below +8 ºC. Keep out of reach of children. Keep away from food, drink and animal feeding stuffs.

Transport: Not classified as dangerous in the meaning of transport regulations.

Source: Rotstop safety data sheet, Verdera 2004

B.3.1.8 Procedures for destruction or decontamination (Annex IIB 3.8)

Technical grade of the MPCA is a hypothetical stage of a continuous production process and no MSDS for the active substance is available. A MSDS for the representative formulation Rotstop is available:

Disposal considerations: Packagings can be landfilled or incinerated, when in compliance with local and national regulations. Wastes can be landfilled, when in compliance with local and national regulations.

Source: Rotstop safety data sheet, Verdera 2004

B.3.1.9 Measures in case of an accident (Annex IIB 3.9)

Technical grade of the MPCA is a hypothetical stage of a continuous production process and no MSDS for the active substance is available. A MSDS for the representative formulation Rotstop is available:

Personal precautions: Avoid contact with skin, eyes and clothing. Use personal protective equipment.

Environmental precautions: The active ingredient is a common naturally occurring fungus and it is not known to have adverse effects on the environment.

Methods for cleaning up: Use personal protective equipment. Pick-up and arrange disposal without creating dust. Shovel into suitable container for disposal. Dispose of in compliance with local and national regulations.

Source: Rotstop safety data sheet, Verdera 2004

B.3.2 Data on application (Annex IIIM 3)

B.3.2.1 Field of use envisaged (Annex IIIB 3.1)

Phlebiopsis gigantea, the active substance of Rotstop, is used to control root and butt rot in conifers caused by the *Heterobasidion annosum* complex. Currently Rotstop is used for the control of *H. annosum* in pine species (mainly Scots pine and Corsican pine) and spruce species (mainly Norway spruce). Future use may include other conifer species such as Sitka spruce, Douglas fir, European larch etc, depending on the outcome of on-going and future efficacy trials.

B.3.2.2 Mode of action (Annex IIIB 3.2)

Pathogenic fungus *Heterobasidion annosum sensu lato* is a white-rot fungus widely distributed in coniferous forests in the Northern Hemisphere. In addition to the European members of the complex (which were previously *H. annosum*- sub-species known as the P-type, S and E-type, but are now considered as separate species - *H. annosum s. stricto*, *H. parviporum* and *H. abietinum*). American groups have also been identified. Spreading of the fungal disease in coniferous forest stands is prevented by treating fresh stumps in thinnings and clear-fellings with a spore suspension of *P. gigantea*.

P. gigantea is able to prevent colonisation of stumps by *H. annosum* through competition for resources. Although there may be a degree of hyphal interference of *H. annosum* by *P. gigantea*, there is no evidence in the available literature that *P. gigantea* controls *H. annosum* by antibiotic or toxic means (Meredith 1960; Rishbeth 1963; Gremmen 1963; Ikediugwu et a. 1970; Ikediugwu 1976; Capretti & Mugnai 1989; Holdenrieder & Greig 1998).

B.3.2.3 Details of intended use (Annex IIIB 3.3)

Table B.3.2.3.a Summary of intended uses of *Phlebiopsis gigantea*

Crop and/ or situation	Member State or Country	Product name	F G or I	Pests or Group of pests controlled	Formulation		Application				Application rate per treatment			PHI days	Remarks
					Type	Conc. of MPCA	Method Kind	Growth stage & season	Number min max	Interval between applications	kg MPCA/hL min max	water L/ha min max	kg MPCA/ha cfu MPCA/ha min max		
(a)			(b)	(c)	(d-f)	(I)	(f-h)	(j)	(k)					(l)	(m)
Pine and spruce forests	Northern and Central Europe	Rotstop	F	*Heterobasidion annosum* and *Heterobasidion parviporum*	WP	$2x10^6$ - 10^7 cfu/g, 10 % (w/w)	Mechanised or manual spraying of freshly cut stumps	First thinning to final cutting, all year at temp's above 5 °C	Once per harvesting time	Minimum 10-15 years in the same stand	100 mg/L	1 L/m^2 stump surface in manual treatment, 2 L/m^2 stump surface in mechanised treatment	100-200 mg/m^2 stump surface, equivalent to 0.8-1.6 g/ha in first thinnings and 3.4-6.8 g/ha in final cutting Min $8x10^6$ cfu/ha Max $1.4x10^9$ cfu/ha	NA	Spraying of the stump surface only, with minimized application around the stump

Remarks:
(a) The EU classification for crops (90/642/EEC).
(b) Outdoor or field use (F), glasshouse application (G) or indoor application (I)
(c) eg. biting and sucking insects, soil bourn insects, foliar fungi, weeds
(d) eg. wettable powder (WP), emulsifiable concentrate (EC), granule (GR)
(e) GCPF Codes - GIFAP Technical Monograph No 2, 1989
(f) All abbreviations used must be explained
(g) Method, eg. high volume spraying, low volume spraying, spreading, dusting, drench
(h) Kind, eg. overall, broadcast, aerial spraying, row, individual plant, between the plants - type of equipment used
(i) g/kg, g/l or appropriate term for micro-organisms
(j) Growth stage at last treatment (BBCH Monograph, Growth Stages of Plants, 1997, Blackwell, ISBN 3-8263-3152-4), including where relevant, information on season at time of application
(k) The minimum and maximum number of applications possible under practical conditions of use must be provided
(l) PHI - minimum pre-harvest interval
(m) Remarks may include: Extent of use/economic importance/restrictions

B.3.2.4 Application rate (Annex IIIB 3.4)

Crop	Method of application	Rate of application per unit treated (as preparation)	Rate of application per unit treated (as active substance)
Pine and spruce	Mechanised or manual spraying of freshly cut stumps	1-2 g Rotstop/m^2 stump surface	100-200 mg or $2x10^6$-$2x10^7$ cfu of *Phlebiopsis gigantea*/m^2 stump surface

B.3.2.5 Content of micro-organism in material used (e.g., in the diluted spray, baits or treated seed) (Annex IIIB 3.5)

Crop	Method of application	Material used (e.g. diluted spray, baits, treated seed)	Content of microorganism in material used
Pine and spruce	Mechanised or manual spraying of freshly cut stumps	Diluted spray	100 mg *Phlebiopsis gigantea*/l or $2x10^6$ -10^7 cfu/l

B.3.2.6 Method of application (Annex IIIB 3.6)

Crop	Method of application	Type of equipment used	Type and volume of diluent per unit of area or volume
Pine and spruce	Mechanised or manual spraying of freshly cut stumps	In manual treatment: a backpack sprayer or a spray bottle In mechanised treatment: application through the sawbar or by a sprayer	1 L/m^2 stump surface in manual treatment, 2 L/m^2 stump surface in mechanised treatment

B.3.2.7 Number and timing of applications (Annex IIIB 3.7)

Crop	Method of application	Maximum number of applications	Timing of application
Pine and spruce	Mechanised or manual spraying of freshly cut stumps	Once per harvesting time	First thinning to final cutting, all year at temp's above 5 ^oC

B.3.2.8 Necessary waiting periods or other precautions to avoid phytopathogenic effects on succeeding crops (Annex IIIB 3.8)

Crop	Method of application	Duration of protection afforded by each application
Pine and spruce	Mechanised or manual spraying of freshly cut stumps	Not relevant

B.3.2.9 Proposed instructions for use (Annex IIIB 3.9)

The Rotstop treatment instructions are the following:

1. The stumps should be treated at felling or within 3 hours from felling
2. in the manual treatment operator should use a backpack sprayer or a spray bottle and spray a layer of the suspension about 1 mm thick evenly over the stump surface

3. in mechanized treatment is average 2 litres of prepared Rotstop suspension per sq. metre of stump surface

4. Treatment in mechanized harvesting:

4.1 Application should be through the sawbar or by a sprayer

4.2 A 25 gram bag of Rotstop is sufficient for an area yielding between 40-80 cubic metres of harvested timber

4.3 A 100 gram bag of Rotstop is sufficient for an area yielding between 150-300 cubic metres of harvested timber

4.4 The equipment used in spreading needs to be cleaned at least once a week

The preparation of Rotstop suspension:

1. A corner of the bag should be cut off and should be poured 0.3 litres of water into a 25 gram bag or 0.6 litres of water into a 100 gram bag. A clean cold or lukewarm water should be used.

2. The cut corner should be fold over and shaked the contents carefully until the powder and water have formed an even suspension

3. The suspension should be mixed into more water (1 litre per 1 gram of powder) e.g. the contents of a 25 gram bag into 25 litres of water

4. The bag should be rinsed and rinsing water should be added to the suspension

5. A Turf Mark dye tablet should be dissolved into the suspension (1 tablet/25-100 litres of suspension) to help monitoring the evenness of spreading. The tablet dissolves entirely in about 15 minutes.

6. The suspension should be used within 24 hours

7. Personal protective equipment should be used when handling Rotstop: overalls and protective footwear, rubber or plastic gloves, head covering, when preparing the suspension respirator equipped with P2 dust filter should be used.

B.3.3 Further information on the plant protection product (Annex IIIB 4)

B.3.3.1 Packaging and compatibility of the preparation with proposed packaging materials (Annex IIIB 4.1)

Rotstop is packed in Polyguard LL-CC/EVOH/PE laminate paper bags (Amcor Flexibles).

Package sizes are 2, 5, 25 and 100 grams. A package specimen is provided.

The suitability of the packaging material for Rotstop was checked in a shelf-life study and was found to maintain product quality according to the quality criteria. Packaged in this material, Rotstop maintained its viability during one month at 28^{o}C and one year at 4^{o}C. No decrease in viability was observed after more than one year at freezer temperature (Palin-Holmberg 2002).

B.3.3.2 Procedures for cleaning application equipment (Annex IIIB 4.2)

There are no label instructions regarding cleaning of equipment and protective clothing.

Equipment and protective clothing are cleaned with water.

B.3.3.2.1 Effectiveness of the cleaning procedures

No data.

B.3.3.3 Re-entry periods, necessary waiting periods or other precautions to protect man, livestock and the environment (Annex IIIB 4.3)

No waiting periods required.

B.3.3.4 Recommended methods and precautions concerning: handling, storage, transport or fire (Annex IIIB 4.4)

A MSDS for the representative formulation Rotstop is available:

Handling: Avoid contact with skin, eyes and clothing. Do not breath dust. Wear personal protective clothing, effective dust mask and gloves. Wash hands and face with soap and water before breaks and after handling the product.

Storage: Keep unopened in a dry and cool place at temperatures below +8 ºC. Keep out of reach of children. Keep away from food, drink and animal feeding stuffs.

Transport: Not classified as dangerous in the meaning of transport regulations.

Fire: Not relevant.

Source: Rotstop safety data sheet, Verdera 2004

B.3.3.5 Measures in the case of an accident (Annex IIIB 4.5)

A MSDS for the representative formulation Rotstop is available:

Containment of spillages:	Not relevant since the active ingredient is a common, naturally occurring fungus.
Decontamination of areas, vehicles and buildings:	Not relevant since the active ingredient is a common, naturally occurring fungus.
Disposal of damaged packaging, adsorbents and other materials:	Arrange disposal without creating dust. Packaging and wastes can be landfilled or incinerated when in compliance with local and national regulations.
First aid measures upon inhalation:	Remove from exposure to fresh air. Obtain medical attention.
First aid measures following skin contact:	Wash off immediately with soap and plenty of water. If skin irritation persists, call a physician.
First aid measures following eye contact:	Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.
First aid measures upon swallowing:	Immediately give plenty of water. Never give anything by mouth to an unconscious person. Induce vomiting and call a physician.

Source: Rotstop safety data sheet, Verdera 2004

B.3.3.6 Procedures for destruction or decontamination of the plant protection product and its packaging (Annex IIIB 4.6)

A MSDS for the representative formulation Rotstop is available:

Disposal considerations: Packagings can be landfilled or incinerated, when in compliance with local and national regulations. Wastes can be landfilled, when in compliance with local and national regulations.

Source: Rotstop safety data sheet, Verdera 2004

B.3.4 References relied on

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
Annex II Data and Information					
IIB 3.5	Meredith, D.S.	1960	Further observations on fungi inhabiting pine stumps. Ann. Bot. Lond.(n.s), Vol. 24 (93), pp. 63-78. Not GLP. Published.	N	
IIB 3.5	Rishbeth, J	1963	Stump protection against *Fomes annosus*. III. Inoculation with *Peniophora gigantea.* Ann. Appl. Biol. Vol. 52 (1), pp. 63 – 77. Not GLP. Published.	N	
IIB 3.5	Gremmen, J	1963	Biological control of the root-rot fungus *Fomes annosus* (Fr.) Cke by *Peniophora gigantea* (Fr.) Masse. Med Barbouw. Ned. Bosb. Tijdschr. Vol. 35(9), pp. 356-367. Not GLP. Published.	N	
IIB 3.5	Ikediugwu, F.E.O., Dennis, C., Webster, J.	1970	Hyphal interference by *Peniophora gigantea* and *Heterobasidion annosum*. Trans. Br. Mycol. Soc., Vol. 54 (2), pp. 307 – 309. Not GLP. Published.	N	
IIB 3.5	Ikediugwu, F.E.O.	1976	The interface in hyphal interference by *Peniophora gigantea* against *Heterobasidion annosum.* Trans. Br. Mycol. Soc. Vol. 66, pp. 291 - 296. Not GLP. Published.	N	
IIB 3.5	Capretti, P., Mugnai, L.	1989	*In vitro* test of antagonism against *Heterobasidion annosum* (Fr.) Bref. Phytopath. Medit. Vol. 28, pp. 155 – 157. Not GLP. Published.	N	
IIB 3.5	Holdenrieder, O., Greig, B.J.W	1998	Biological methods of control. In: Woodward *et al.* (eds). *Heterobasidion annosum*. Biology, Ecology, Impact and Control. CAB International, UK, pp. 235 – 258. Not GLP. Published.	N	
IIB 3.5	Korhonen, K.	2003a	Identification of fungal isolates from the biopreparates 1984, 1985 and 1986, made by Verdera Oy for treating conifer stumps against *Heterobasidion.* Report. Finnish Forest Research Institute. 2 pp. Not GLP. Unpublished.	Y	VRA

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
IIB 3.5 IIB 3.6	Korhonen, K.	2003b	Simulated stump treatment experiments for monitoring of the efficacy of *Phlebiopsis gigantea* against *Heterobasidion.* In: Laflamme *et al*. (eds.). Root and butt rots of forest trees. Proc. 10th Int. Conf. on Root and Butt Rots, Quebec City, Canada, 2001, pp. 206-210. Not GLP. Published.	N	
IIB 3.5	Korhonen, K.	2005	Results of billet experiments and stump treatment experiments including *P. gigantea* isolates from Sweden, Canada and UK. Finnish Forest research Institute. Report, 5 pp. Not GLP. Unpublished.	Y	VRA
IIB 3.5	Korhonen, K., Kauppila, P.	1988	The sexuality of *Phlebiopsis gigantea* Karstenia. Vol. 27, pp. 23 – 30. Not GLP. Published.	N	
IIB 3.5	Korhonen, K., Lipponen, K., Bendz, M, Johansson, M., Ryen, I., Venn, K. Seiskari, P., Niemi., M.	1994	Control of *Heterobasidion annosum* by stump treatment with Rotstop - a new commercial formulation of Phlebiopsis gigantea. Proc. 8t Int. Conf. Root and butt rots, Sweden & Finland 1993, pp. 675 – 685. SUAS, Uppsala. Not GLP. Published.	N	
IIB 3.5	Korhonen, K., Stenlid, J.	1998	Biology of *Heterobasidion annosum*. In: Woodward *et al*. (eds). *Heterobasidion annosum*. Biology, Ecology, Impact and Control. CAB International, UK, pp. 43 – 70. Not GLP. Published.	N	
IIB 3.6	Hallaksela, A-M., Korhonen, K.	1992a	Identification of the fungus from the biopreparate made by Kemira Oy for conifer stump treatment. Report. Finnish Forest Research Institute. 4 pp. Not GLP. Unpublished.	Y	VRA
IIB 3.6	Seiskari, P.	2005a	SOP 144-1. Maintenance and subculturing of microbial strains used in Rotstop production. Verdera Oy. 3pp. Not GLP. Unpublished.	Y	VRA
IIB 3.6	Seiskari, P.	2005c	SOP 147-1. Rotstop test on agar plates. Verdera Oy. 4 pp. Not GLP. Unpublished.	Y	VRA
IIB 3.6	Thorpe, K.	2005a	SOP: Method for selection of isolates for PG Suspension. Forest Research, UK. Not. GLP. Unpublished.	Y	FOC
IIB 3.7 IIB 3.8 IIB 3.9	Anonymous	2004	Rotstop safety data sheet	Y	VRA

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
Annex III Data and Information					
IIIB 3.2	Meredith, D.S.	1960	Further observations on fungi inhabiting pine stumps. Ann. Bot. Lond.(n.s), Vol. 24 (93), pp. 63-78. Not GLP. Published.	N	
IIIB 3.2	Rishbeth, J	1963	Stump protection against *Fomes annosus*. III. Inoculation with *Peniophora gigantea.* Ann. Appl. Biol. Vol. 52 (1), pp. 63 – 77. Not GLP. Published.	N	
IIIB 3.2	Gremmen, J	1963	Biological control of the root-rot fungus *Fomes annosus* (Fr.) Cke by *Peniophora gigantea* (Fr.) Masse. Med Barbouw. Ned. Bosb. Tijdschr. Vol. 35(9), pp. 356-367. Not GLP. Published.	N	
IIIB 3.2	Ikediugwu, F.E.O., Dennis, C., Webster, J.	1970	Hyphal interference by *Peniophora gigantea* and *Heterobasidion annosum*. Trans. Br. Mycol. Soc., Vol. 54 (2), pp. 307 – 309. Not GLP. Published.	N	
IIIB 3.2	Ikediugwu, F.E.O.	1976	The interface in hyphal interference by *Peniophora gigantea* against *Heterobasidion annosum.* Trans. Br. Mycol. Soc. Vol. 66, pp. 291 - 296. Not GLP. Published.	N	
IIIB 3.2	Capretti, P., Mugnai, L.	1989	*In vitro* test of antagonism against *Heterobasidion annosum* (Fr.) Bref. Phytopath. Medit. Vol. 28, pp. 155 – 157. Not GLP. Published.	N	
IIIB 3.2	Holdenrieder, O., Greig, B.J.W	1998	Biological methods of control. In: Woodward *et al.* (eds). *Heterobasidion annosum*. Biology, Ecology, Impact and Control. CAB International, UK, pp. 235 – 258. Not GLP. Published.	N	
IIIB 4.1	Palin-Holmberg, G.	2001/ 2002	Shelf life test for biocontrol products in paper-EVOH-laminate. Test report 01244-ETs. Kemira Oyj, Espoo Research Centre. 7 pp. Not GLP. Unpublished.	Y	VRA
IIIB 4.4 IIIB 4.5 IIIB 4.6	Anonymous	2004	Rotstop safety data sheet	Y	VRA

**: Protection for 5 years claimed from date of decision concerning listing in Annex I - the study report has not been submitted any of the Member States in support of an application for authorization, or (though the study report has been submitted) has not been used any of the Member States as the basis for decision on the initial authorization, or to maintain a given authorization, of a plant protection product before the date of submission of the dossier to Rapporteur Member State.*

***: Owners' code identifications and names (Code identification: VRA – Verdera; FOC – Forestry Commission)*

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Volume 3

Annex B.4

Proposals for classification and labelling

Rapporteur Member State: Estonia

April 2007

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B.4 Proposals for classification and labelling

B.4.1 Proposals for classification and labelling of the MPCA

Technical grade of the MPCA is a hypothetical stage of a continuous production process and no MSDS for the active substance is available.

B.4.2 Proposals for classification and labelling of the MPCP

A MSDS for the representative formulation Rotstop is available:

Classification with regard to physical/chemical data:	No classification
Classification with regard to toxicological data:	S2, S13
Classification with regard to fate and behaviour:	No classification
Classification with regard to ecotoxicological data:	No classification
Information on safe handling:	Keep out of reach of children. Keep away from food, drink and animal feeding stuffs. Safety precautions: Use protective clothing and shoes, rubber or plastic (e.g. nitrile) gloves and a cap when handling the preparate. Use also a half mask with dust filter P2 when preparing the working solution. Environmental precautions: Empty packages can be disposed of with household waste.
Information on storage:	Store in dry and cool conditions. An unopened package stored below +8°C remains active for 12 months and at –18°C for 18 months. A package taken from cool conditions should be used within one week. An opened package and the working solution should be used within one day.
Names of the ingredients given on the warning label:	Dried spores and mycelium of *Phlebiopsis gigantea*.

Source: Rotstop safety data sheet, Verdera, 2003

RMS comments: Justified proposals for classification and labelling of Rotstop according to Directive 67/548/EEC and Commission Directive 2001/36/EC are listed below.

Hazard symbol : Xi

Indication of danger : Irritant

Risk phrases : R43 May cause sensitisation by skin contact

Safety phrases : S2 Keep out of the reach of children

: S13 Keep away from food, drink and animal feeding stuffs

: S22 Do not breathe dust

: S23 Do not breathe gas/fumes/vapour/spray

: S24 Avoid contact with skin

: S25 Avoid contact with eyes

: S37 Wear suitable gloves

: S42 During spraying wear suitable respiratory equipment

Justification for the proposal:

R43: Sensitisation studies are considered not suitable for micro-organisms, also, negative results in a Buehler test are not acceptable, since this test is considered less sensitive. According to the directive 2001/36/EC all micro-organisms should be regarded as potential sensitiser.

S2: Obligatory for preparations sold to the general public

S13: Recommended when substances and preparations are likely to be used by general public

S22/23: Required for substances with R42

S24/37: Required when it is necessary to draw the attention of the user to skin contact risks

S25: Recommended when it is necessary to draw the attention of the user to eye contact risk

S42: The formulation is described as a fine powder and therefore there could be the potential for inhalation from dust.

B.4.3 References relied on

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
Annex II Data and Information					
Annex III Data and Information					
	Anonymous	2003	Rotstop safety data sheet	Y	VRA

**: Protection for 5 years claimed from date of decision concerning listing in Annex I - the study report has not been submitted any of the Member States in support of an application for authorization, or (though the study report has been submitted) has not been used any of the Member States as the basis for decision on the initial authorization, or to maintain a given authorization, of a plant protection product before the date of submission of the dossier to Rapporteur Member State.*

***: Owners' code identifications and names :*VRA – Verdera;

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Volume 3

Annex B.5
Analytical methods

Rapporteur Member State: Estonia

April 2007

TAIMETOODANGU INSPEKTSIOON

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B.5 Analytical methods (Annex IIB 4)

B.5.1 Methods for the analysis of the micro-organism as manufactured and for the analysis of the preparation (Annex IIB 4)

B.5.1.1 Methods for the analysis of the micro-organism as manufactured (Annex IIB 4.1)

B.5.1.1.1 Methods for the identification of the micro-organism (Annex IIB 4.1.1)

The same standard microbiological methods can be used for all *P. gigantea* strains to identify *P. gigantea* strains on species level.

Identification on species level using classical taxonomy based on morphology and growth characteristics of the fungus (growth rate, microscopic appearance of mycelium and conidial structures, enzyme activity etc.). Identification on strain level based by using molecular identification methods e.g. RAPD and RAMS/PCR and strain-specific markers.

Phlebiopsis gigantea is morphologigally described in DSMZ study (Braunschweig, september 24, 2004): Colonies of Phlebiopsis gigantea are growing rapidly on malt-extract agar (Petri dish size 90 mm Ø, temperature 25 ° C, 5 days); mycelium hyaline, at first appressed, later later farinaceous. Colony reverse pale: unchanged; laccase reaction with gualacol negative. No fruiting structures observed. Hyphal diameter variable (4-8 µm); no skeletal or binding hyphae; clamp connections present only on few submerged hyphae, seldom double or multiple. Aerial mycelium partially disintegrating to form numerous cylindrical arthrospores. Chiamydospores and other conidia absent.

Table B.5.1.1.1 Methods for the identification of different strains of *Phlebiopsis gigantea*.

Strain no.	Molecular identification method	Morphological identification criteria	Reference
VRA 1835	RAMS-PCR analysis	Growth characteristics of mycelium and spores. Enzyme activity.	Hallaksela & Korhonen (1992a) Hoffman (2004) Vainio *et al*. (2001)
VRA 1984	ITS-sequence analysis	Mycelial characteristics.	Korhonen (2003a) Holmer & Stenlid (2003)
VRA 1985	ITS-sequence analysis	Mycelial characteristics.	Korhonen (2003a) Holmer & Stenlid (2003)
VRA 1986	ITS-sequence analysis	Mycelial characteristics.	Korhonen (2003a) Holmer & Stenlid (2003)
FOC PG B20/5	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG SP log 6	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG SP log 5	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)

FOC PG BU 3	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG BU 4	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG 410.3	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG97/1062/116/1.1	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG B22/SP1287/3.1	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG SH 1	PCR-RAPD analysis	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)
FOC PG B22/SP1190/3.2	Molecular identification in progress	Growth characteristics of mycelium and spores	Thorpe (2005a) Webber & Thorpe (2003)

B.5.1.1.2 Methods for providing information on possible variability of seed stock/active micro-organism (Annex IIB 4.1.2)

In order to ensure the consistency and integrity of the master and seed stock, the *P. gigantea* strains are preserved under liquid nitrogen, at –80°C and at 4°C.

Typical features of the strains such as growth rate, growth characteristics of the mycelium and fecundity (oidial production) are continuously monitored in order to reveal any major changes in the behaviour of the strain. Also the ligninolytic enzyme activity of a strain and its competitive behaviour in dual culture with *Heterobasidion annosum* and *H. parviporum* can be assessed at intervals.

The following procedures are used for the production of the *P. gigantea* strains containing formulations:

Reference: Seiskari, P. (2005a): SOP 144-1. Maintenance and subculturing of microbial strains used in Rotstop production. Verdera Oy. 3pp.

Not GLP. Unpublished.

Summary:

- seed bank ampoules, containing spores of the strain harvested from malt agar plates, are stored under liquid nitrogen in Verdera's culture collection
- for each strain, a general supply of ampoules is stored in -80°C; a batch of 100 ampoules containing spore suspension is prepared from one seed bank ampoule in the same way as the seed bank ampoules; each batch is checked for viability and visual inspection of growth morphology
- inoculum for production is prepared by plating spore suspension from a general supply ampoule on potato dextrose agar.

Reference: Thorpe, K. (2005a): SOP: Method for selection of isolates for PG Suspension. Forest Research, UK.

Not. GLP. Unpublished.

Summary: This SOP covers the steps involved in the selection of isolates of the fungus *Phlebiopsis gigantea* for use in the alternative formulation PG Suspension. However, some of the *in vitro* tests outlined are also used to

quantify the performance of isolates already in use in PG Suspension. This ensures the consistency and integrity of the master and working seed stock is maintained, as isolates can degrade in culture. *In vitro* growth rates on wood discs and artificial media are performed, in addition to quantification of oidia production on Phostrogen agar.

Reference: Hallaksela, A-M., Korhonen, K. (1992a): Identification of the fungus from the biopreparate made by Kemira Oy for conifer stump treatment. Report. Finnish Forest Research Institute. 4 pp.
Not GLP. Unpublished.

Summary: Monitoring of the growth characteristics of *P. gigantea* in agar culture is part of the routine quality control in production, and includes visual assessment of the amount and appearance of the aerial mycelium (incrusted hyphae, a feature typical for this species) and the extent of sporulation (formation of oidial spore chains). Major changes in the appearance of the fungus in agar culture may be an indication of strain degeneration.

Reference: Hallaksela, A-M., Korhonen, K. (1992b): Isolation of *P. gigantea* from a tree stump or log. Finnish Forest Research Institute. 1 p.

Not GLP. Unpublished.

Summary: *P. gigantea* infection in pine and spruce wood can be detected on the basis of the typical red-brownish rot it causes, which is easy to recognize. Such an area can be sampled aseptically and a small piece of wood plated on malt agar containing benomyl to prevent the growth of mould fungi. After incubation at room temperature, the fungus growing from the wood sample can be identified based on its morphological characteristics.

Reference: Seiskari, P. (2005c): SOP 147-1. Rotstop test on agar plates. Verdera Oy. 4 pp.

Not GLP. Unpublished.

Summary: Characteristics of *P. gigantea* that are important for the control the efficacy against *H. annosum* are mycelial growth rate and wood colonising ability, both contributing to the competitive ability of *P. gigantea* to rapidly establish in the stump. A major change in either of these can be detected using a simple test, where the mycelial growth increment on malt agar is measured daily and the ligninolytic activity of the isolate is determined as the ability to degrade a lignin-derivative dye (Remazol Brilliant Blue) added to the malt agar. In addition, the competitive ability of *P. gigantea* can be assessed as the extent to which it overgrows the mycelium of *H. annosum* in dual culture on malt agar plates. The test can also be done with pine or spruce sawdust instead of malt extract as the sole carbon source in the agar.

Reference: Korhonen, K (2003b): Simulated stump treatment experiments for monitoring the efficacy of *Phlebiopsis gigantea* against *Heterobasidion.* In: Laflamme *et al.* (eds.). Root and butt rots of forest trees. Proc. 10th Int. Conf. on Root and Butt Rots. Quebec City, Canada, 2001, pp 206-210.

Not GLP. Published.

Summary: Simulated stump treatment experiments can be used to monitor the efficacy of *P. gigantea* in protecting stumps from *H. annosum* infection. One half of the upper surface of freshly cut 20-30 cm long pieces of pine or spruce stems is treated with a *P. gigantea* spore suspension solution, and ½-1 hour later the whole surface is sprayed with a conidial suspension of *Heterobasidion.* The stem pieces are kept standing on moist sand in the open air or in a glasshouse, and after 6-8 weeks sample discs are cut from the logs, incubated for a week in plastic bags and checked for the occurrence of *Heterobasidion* conidiophores under a dissecting microscope. The efficacy of the treatment is calculated by comparing the areas occupied by *H. annosum* on the two halves of each log piece. During these standardised test conditions with artificial *H. annosum* inoculation, a change in any characteristics of major importance for the control the efficacy of the *P. gigantea* strain will be revealed.

B.5.1.1.3 Methods to differentiate a mutant of the micro-organism from the parent wild strain (Annex IIB 4.1.3)

Not relevant since all the strains of *P. gigantea* are wild isolates and are not genetically modified in any way.

B.5.1.1.4 Methods for the establishment of purity of seed stock from which batches are produced and methods to control that purity (Annex IIB 4.1.4)

In order to ensure the consistency and integrity of the master and seed stock, the *P. gigantea* strains are preserved under liquid nitrogen, at –80°C and at 4°C.

Typical features of the strains such as growth rate, growth characteristics of the mycelium and fecundity (oidial production) are continuously monitored in order to reveal any major changes in the behaviour of the strain. Also the ligninolytic enzyme activity of a strain and its competitive behaviour in dual culture with *Heterobasidion annosum* and *H. parviporum* can be assessed at intervals.

The following procedures are used for the production of the *P. gigantea* strains containing formulations:

Reference: Seiskari, P. (2005a): SOP 144-1. Maintenance and subculturing of microbial strains used in Rotstop production. Verdera Oy. 3pp.

Not GLP. Unpublished.

Summary:

- seed bank ampoules, containing spores of the strain harvested from malt agar plates, are stored under liquid nitrogen in Verdera's culture collection

- for each strain, a general supply of ampoules is stored in -80°C; a batch of 100 ampoules containing spore suspension is prepared from one seed bank ampoule in the same way as the seed bank ampoules; each batch is checked for viability and visual inspection of growth morphology

- inoculum for production is prepared by plating spore suspension from a general supply ampoule on potato dextrose agar.

Reference: Thorpe, K. (2005a): SOP: Method for selection of isolates for PG Suspension. Forest Research, UK.

Not. GLP. Unpublished.

Summary: This SOP covers the steps involved in the selection of isolates of the fungus *Phlebiopsis gigantea* for use in the alternative formulation PG Suspension. However, some of the *in vitro* tests outlined are also used to quantify the performance of isolates already in use in PG Suspension. This ensures the consistency and integrity of the master and working seed stock is maintained, as isolates can degrade in culture. *In vitro* growth rates on wood discs and artificial media are performed, in addition to quantification of oidia production on Phostrogen agar.

B.5.1.1.5 Methods to determine the content of the micro-organism in the manufactured material used for the production of formulated products and methods to show that contaminating micro-organisms are controlled to an acceptable level (Annex IIB 4.1.5)

The same standard microbiological methods can be used for all *P. gigantea* strains supported in this DAR. In practice there are some differences in choice of methods, depending on which product formulation the strain is used for.

Depending on the type of formulation, the concentration of micro-organism in the formulated product is $2x10^6$-10^7 cfu of *P. gigantea*/g for a wettable powder WP (nominal 5 x 10^6 cfu/g) and 3.5 $x10^6$-10^7 cfu of *P. gigantea*/ml for a suspension concentrate (SC).

The representative formulation Rotstop contains 8-12 % w/w of *P. gigantea* (nominal 10 % w/w). The alternative formulation PG Suspension contains <0.5 % w/w of *P. gigantea*.

Reference: Seiskari ,P. (2004): SOP 101-1. Viability determination of biological pesticides. Verdera Oy, 4 pp.

Not GLP. Unpublished.

Summary: A standard dilution-plate counting method is used to determine the viability of biopesticide samples, and it can be used to determine the amount of viable micro-organisms in other sample matrices as well. For enumeration of *P. gigantea* the sample is mixed with water and homogenised, a dilution series is prepared and a known volume of each dilution is spread on potato dextrose agar plates. The plates are incubated until the colonies are countable (28 ºC, approximately 2 days), and the number of viable propagules (colony forming units, cfu) is calculated on the basis of colony counts from the different dilution steps.

Reference: Seiskari, P. (2005b): SOP 120-2 Viability determination of biological pesticides using MPN-method. Verdera Oy. 4 pp.

Not GLP. Unpublished.

Summary: For microbes that do not form distinct colonies, such as the fungus *P. gigantea,* a Most Probable Number (MPN) method for the determination of the viability has been found easier to perform and more accurate. In the MPN-method, a series of water dilutions is prepared and a known volume of each dilution is pipetted on several spots on potato dextrose agar plates. The plates are incubated (28 ºC, approximately 4…6 days) and the positive and negative spots are counted to give the most probable number of viable units in the original sample.

Reference: Thorpe, K. (2005a): SOP: Method for selection of isolates for PG Suspension. Forest Research, UK.

Not. GLP. Unpublished.

Summary: Fruiting-bodies are frequently formed on stumps that have been treated with a strain of *P. gigantea*. The strain can be isolated from the wood under such a fruiting body by aseptically removing a small chip of colonized wood and incubating it on malt antibiotic thiabendazole agar, which is a selective medium for fungi belonging to the Basidiomycotina. After 2-5 days incubation, the strain can be identified based on its morphological characteristics.

To determine the fecundity of *P. gigantea* isolates, spore solutions are made up by shaking colonised nutrient-rich agar plugs in water. The numbers of spores (in reality a mixture of oidia and mycelial fragments) within the resulting solution are quantified by pipetting samples onto a haemocytometer – a glass slide with a grid etched onto the surface - and examining the spore numbers under magnification. By counting the numbers of spores within each square on the grid, an estimate can be made of the overall spore concentration in the original solution. The fecundity of different isolates can then be compared.

Reference: Seiskari, P. (2005h): SOP 143-1. Quality control of Rotstop production. Verdera Oy. 3 pp.
Not GLP. Unpublished.

Information about this study is available in Confidential information, Document C

B.5.1.1.6 Methods for the determination of relevant impurities in the manufactured material (Annex IIB 4.1.6)

Two methods are used in the production of end-products containing any of the 14 strains of *P. gigantea* supported in this dossier. The two production methods result in different formulations, but both are aseptic, continuous manufacturing processes where cultivation conditions are adjusted for optimal growth of the fungus in order to yield acceptable quality in the end-product. Control to a specified and acceptable level of microbial impurities is achieved by following a strict quality control protocol throughout the manufacturing process. Some of the procedures are described below, detailed information about the production methods and quality control are given in Confitential information, Document C.

Reference: Seiskari, P. (2005d): SOP 163-3. Quality control in Rotstop production facilities. Verdera Oy. 1 p.

Not GLP. Unpublished.

Summary: The hygiene of the production facilities is maintained by standard procedures, including overpressure, sterile filters, disinfection, UV lights etc. The production process for Rotstop is aseptic and semi-aseptic, except for the final stages of milling and packaging of the dry product.

Reference: Seiskari, P. (2005h): SOP 143-1. Quality control of Rotstop production. Verdera Oy. 3 pp. Not GLP. Unpublished.

Information about this study is available in Confidential information, Document C

Reference: Lahdenperä, M-L. (2005): SOP 165-1. Method for surveying viable microbes in the indoor air in the production facilities of Rotstop. Verdera Oy. 1 p.

Not GLP. Unpublished.

Summary: Occasional surveys of the amount and kind of microbes present in the air give qualitative information of the hygiene level in the production facilities. The surveys are done by placing agar plate traps in different parts of the production facilities, and identifying the present contaminants by microscopy.

Contamination is monitored throughout the production process by visual inspection and plate dilution of samples, and contaminated batches are discarded. The amount of contaminants in the end-product is determined quantitatively using either of the methods described in point B.5.1.1.5.

P. gigantea does not produce any toxic metabolites, and no method for that purpose is available. No other impurities of toxicological concern are likely to be present at any stage of the manufacturing process.

B.5.1.1.7 Methods to control the absence and to quantify (with appropriate limits of determination) the possible presence of any human and mammalian pathogen (Annex IIB 4.1.7)

Quality control protocols for the production of end-use products are developed to detect and eliminate contamination during the aseptic production process and minimize the amount of contaminants in the packaged end-product. Although unlikely to enter the process at any stage, presence of pathogenic contaminants in the end-product is checked annually in samples from 2–5 random batches, sent to an external independent laboratory for testing.

Reference: Seiskari, P. (2005e): Analysis of pathogenic contaminants in Rotstop. Report 159-1. Verdera Oy. 1 p. + 4 appendices.

GLP Yes. Unpublished.

Summary: Four commercial batches of Rotstop were analysed. The analyses of coliform bacteria and *Salmonella* were done using the standard methods ISO6579 (2002) and NMKL 44 (1995) for total coliforms (including *E. coli*) and *Salmonella*, respectively (formerly used methods are M75901 and M76104). No coliforms or *Salmonella* were detected from the samples.

B.5.1.1.8 Methods to determine storage stability, shelf-life of the micro-organism, if appropriate (Annex IIB 4.1.8)

The same standard microbiological methods can be used for all *P. gigantea* strains. In practice there are some differences in choice of methods, depending on which product formulation the strain is used for.

Table B.5.1.1.8a: Storage stability of biological control agents.

Reference:	Seiskari, P. (2005f): SOP 161-1. Storage stability of biological control agents. Verdera Oy. 1 p. Not GLP. Unpublished.
Results:	Storage stability is determined by analysing the viability of *P. gigantea* stored at different temperatures. Accelerated (short-term) storage stability at 28°C is determined after 1 week and 1 month, and long-term storage stability at 4°C is assessed at regular intervals during 1 year.

RMS comments:	The methods and the results are acceptable.

Table B.5.1.1.8b: Accelerated storage

Reference:	Seiskari, P. (2005j): Storage stability of Rotstop. Report-149-1. Verdera Oy. 2 pp. Not GLP. Unpublished.
Test Material:	Commercial Rotstop batch
Method:	Eight commercial batches of Rotstop were stored in plastic sample holders in a refrigerator at +4 ºC and in incubation chamber at +28 ºC. Viability of samples was analysed according to SOP-101-1 "Viability determination of biological pesticides".
Results:	There was no significant change in the viability of Rotstop or container of the test material during storage at +28 ºC for one month and at +4 ºC at least one year (viability over $2x10^6$ cfu/g).
RMS comments:	The methods and the results are acceptable.

B.5.2 Methods for the analysis of the preparation (Annex IIIB 5.1)

The representative formulation Rotstop has the same formulation as the microbial pest control agent (MPCA), because the end product of production of the MPCA is exactly the required formulation for the MPCP.
For detailed information see point B.5.1.

B.5.2.1 Methods for the identification and the determination of the content of the micro-organism(s) in the preparation

Methods for the identification and determination are described in point B.5.1.

B.5.2.2 Methods to establish regular control of the preparation to show that it does not contain other organisms than the indicated ones and to establish uniformity

Contaminating microbes present in samples taken at various stages of production are detected by using either of the methods described in Point B.5.1.1.5 and B5.1.1.6.

B.5.2.3 Methods to identify any contaminating micro-organisms of the preparation

Contaminating microbes present in samples taken at various stages of production are detected by using either of the methods described in Point B.5.1.1.5 and B5.1.1.6. Colonies appearing on the quality control agar plates are identified based on gross morphology of the colonies and if necessary by more detailed microscopic examination.

The contaminants are usually mould fungi commonly present in the air and can be easily identified by using standard taxonomic identification methods.

B.5.2.4 Methods used to determine the storage stability and shelf life of the preparation (IIIB 5.1.4)

Reference: Seiskari, P. (2005f): SOP 161-1. Storage stability of biological control agents. Verdera Oy. 1 p.

Not GLP. Unpublished.

Summary: Storage stability is determined by analysing the viability of *P.gigantea* after storage of Rotstop at different temperatures. Accelerated (short-term) storage stability at 28°C is determined after 1 week and 1 month, and long-term storage stability at 4°C is assessed at regular intervals during 1 year. The amount of viable propagules in the end-product is determined according to SOP-101 or SOP-140.

Reference: Pulkkanen, H. (1995): Effect of temperature changes during storage and transportation on shelf-life of Rotstop. Memorandum 95093-Esp-Me. Kemira Agro Oy. 3 pp.

Not GLP. Unpublished.

Summary: Storage stability of Rotstop in a simulated logistics chain with alternating warm temperature during transportation and cold temperature during storage was assessed by incubating packages through a temperature cycle simulating the steps from the producer to the end customer. The viability of Rotstop was determined after each incubation step by ordinary dilution-plate counting.

References: Woolley, A.J., Mullee, D.M. (2004a): Determination of accelerated storage stability. SPL Project number: 1841/005. SafePharm Laboratories. 27 pp. Unpublished.

Woolley, A.J., Mullee, D.M. (2004b): Determination of long-term storage stability. SPL Project number: 1841/006. SafePharm Laboratories. 27 pp. Unpublished.

Test Material: Commercial Rotstop batch

Method: The study was conducted according to Methods of the CIPAC Handbook for the Analysis of Technical and Formulated Pesticides Methods of Commission Directive 92/69/EEC and the current OECD Guidelines for testing of Chemicals:

- CIPAC MT75 for pH
- CIPAC MT53.3.2 for wettability
- CIPAC MT47.1 for persistent foaming
- CIPAC MT15.1 for suspensibility
- OECD 110 for dry sieve test and particle size distribution
- CIPAC MT59.3 for wet sieve test
- CIPAC MT33 for tap density

GLP: Yes

Results: There was no significant change in the appearance of the formulation or container of the test material during storage at 28 ± 2 ºC for 7 days and 8 ± 2 ºC for 1 year. See table B.5.2.4.

RMS comments: The methods and the results are acceptable.

Table B.5.2.4: Chemical and physical properties of Rotstop, initially and after 7 days and 1 year of storage

Test	Results		
	Initial	7 days at 28 ± 2 ºC Woolley & Mullee (2004a)	1 year at 8 ± 2 ºC Woolley & Mullee (2004b)
Formulation	Cream, opaque fine powder which formed clumps.	Cream, opaque fine powder which formed clumps. No caking of test material observed.	Cream, opaque fine powder which formed clumps. No caking of test material observed.
Odour (subjective assessment)	Weak fungus like odour	Weak fungus like odour	Weak fungus like odour
Container	Silver opaque foil sachet, with a white manufacturers label attached. No signs of corrosion or degradation.	Silver opaque foil sachet, with a white manufacturers label attached. No signs of corrosion or degradation.	Silver opaque foil sachet, with a white manufacturers label attached. No signs of corrosion or degradation.
Weight change	-	<8.47 x 10^{-3}% (loss/gain)	-
pH at 25 ºC: 1% aqueus dispersion	6.29	6.35	6.30
Particle size distribution: **Percentage less than 100 µm** **Percentage less than 10 µm**	 57.9% 11.3%	 57.6% 11.7%	 68.5% 18.1%
Wettability	115 seconds	129 seconds	286 seconds
Persistent foaming (specified field application concentration 0.1% w/v): **Initial** **10 seconds** **1 minute** **3 minutes** **12 minutes**	 A few bubbles produced around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery.	 A few bubbles produced around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery.	 A few bubbles produced around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery. A few bubbles remained around the periphery.
Suspensibility (specified field application concentration 0.1% w/v)	29.6%	30.0%	22.3%
Wet sieve test	22.1% retained on a 75 µm sieve.	34.0% retained on a 75 µm sieve.	32.9% retained on a 75 µm sieve.

Reference: Seiskari, P. (2002): Sedimentation tests with Rotstop preparations made of Swedish *Phlebiopsis* strains. Test report. Verdera Oy. Unpublished.

GLP:	No
Test Material:	Preparations similar to commercial Rotstop. Preparations made of Swedish *Phlebiopsis gigantea* strains coded 1983, 1984, 1985 and 1986. Also commercial Rotstop.
Method:	Each preparation was crushed both manually in a mortar and with a small laboratory sample mill. Commercial Rotstop milled with a hammer mill. 1 g of each preparation was well mixed with 1 liter of water according to the normal instructions for use. This solution was poured into an Imhoff-cone and left standing. The volume of solids sedimented to the bottom was determined after 5, 15 and 30 minutes. To determine the amount of viable spores suspended in the solution, a sample was taken from the cone about 3 cm below the liquid surface level. Spores were counted using a

	standard plating method: a series of dilutions were plated on potato dextrose agar dishes, the plates were incubated at 28 ºC and colonies were counted after 3 days. The results were compared to the viability of homogenized samples taken before sedimentation.
Results:	Manually crushed preparations sedimented much faster than milled ones. Commercial Rotstop had clearly the best suspensibility. Large particles sedimented during 30 minutes, but the aqueous solution contained sufficient amounts of *P. gigantea* spores to give good protection against *H. annosum*. The viability test shows that the spore concentration in the solution might have decreased slightly when the samples were left standing still for 30 minutes. However, taking into account the inaccuracy of the plating method, about ± 60 %, changes were not drastic and the order of magnitude remained the same.
RMS comments:	The methods and the results are acceptable.

Reference: Seiskari, P. (2005k): Tap density of Rotstop. Report-150-1. Verdera Oy. 1 p.

GLP:	No
Test Material:	Two commercial Rotstop batches: 05565 and 18565.
Method:	Tap density of Rotstop was determined according to CIPAC method MT 33.
Results:	Tap density was 0.22 g/ml.
RMS comments:	The methods and the results are acceptable.

B.5.3 Methods to determine and quantify residues (viable or non-viable) of the micro-organism as manufactured and for the analysis of the preparation (Annex IIB 4.2 and IIIB 5.2)

B.5.3.1 Methods to determine and quantify residues (viable or non-viable) of the micro-organism

Considering that *P. gigantea* is used for the control of the forest fungal pathogen *H. annosum* through stump treatment in coniferous forests, the question of residues is not relevant.

B.5.3.1.1 The active micro-organism(s) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant

Food: Not relevant considering the use of *P. gigantea* as a stump treatment agent in forests.

Feed: Not relevant considering the use of *P. gigantea* as a stump treatment agent in forests.

Animal tissue: Not relevant considering the use of *P. gigantea* as a stump treatment agent in forests.

Soil: Not relevant considering that *P. gigantea* is a primary wood colonising fungus, which does not proliferate in soil.

Water: Not relevant considering that *P. gigantea* is a primary wood colonising fungus, which does not proliferate in water.

Air: The amount of air-borne spores of *P. gigantea* can be determined using agar plates or wood discs as spore traps.

Analytical methods for amount or activity of proteinaceous products: Not relevant for *P. gigantea*.

B.5.3.1.2 Relevant metabolites (especially toxins) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant (IIIB 5)

There is one published study on *P. gigantea*, which reports production of secondary fungal metabolites *in vitro*. However, these were compounds commonly produced by wood-inhabiting fungi or belonging to a class of substances widely distributed in nature. There are no other records of metabolites produced by *P. gigantea* that would be of concern for human health and/or the environment.

Reference: Briggs, L.H., Cambie, R.C., Dean, I.C., Dromgoole, S.H., Fergus, B.J., Ingram, K.G., Lewis, K.G., Small, C.W., Thomas, R. & Walker, D.A. (1975): Chemistry of fungi 10. Metabolites of some fungal species. N. Z. J. Sci. Vol. 18, pp. 565 – 576.

Not GLP. Published.

Summary: In this study metabolites produced by various fungi was investigated in liquid culture, Lup-19(22)-ene and Lupa-15,19(22)-diene were found in the neutral fraction of chloroform extract, and 2',3',5'-trimethoxy-p-terphenyl was detected in the neutral fraction of an ethyl acetate extract of the mycelium. According to Hütterman (1997), this compound is a typical secondary fungal metabolite, and substances like this have been found in almost all wood-inhabiting fungi that have been analysed for the presence of this kind of compounds. This particular compound definitely has a lower toxicity than many other secondary metabolites which have been isolated from fungi against which *P. gigantea* is antagonistic. It is not considered to pose any special harm in the following scenarios: (i) acute toxicity during application, (ii) toxicity on the treated stump, (iii) accumulation in the wood and on the forest floor. Lup-19(22)-ene and Lupa-15,19(22)-diene belong to a class of substances which are widely distributed in nature, e.g. in the bark of trees, in leaves and stems of annual plants, or in seeds. No high toxicity can be expected from these compounds in the case of stump treatment.

There are no other records of metabolites produced by *P. gigantea* that would be of concern for human health and/or the environment (Holdenrieder & Greig, 1998). Studies conducted by Ikediugwu *et al.* (1970) and Capretti & Mugnai (1989) indicate that *P. gigantea* does not depend on the production of toxins for its ability to combat *H. annosum*, but acts through competition for the wood resource.

B.5.3.1.3 Methods to determine and quantify residues (viable or non-viable) of the micro-organism for the analysis of the preparation

Considering that *P. gigantea* is used for the control of the forest fungal pathogen *H. annosum* through stump treatment in coniferous forests, the question of residues is not relevant.

B.5.4 References relied on

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
Annex II Data and Information					
IIB 4.1.1	Hoffman, P.	2004	Identification of fungus cultures. Certificate. DMSZ. Not GLP. Published	N	
IIB 4.1.1 IIB 4.1.5	Hallaksela, A-M., Korhonen, K	1992a	Identification of the fungus from the biopreparate made by Kemira Oy for conifer stump treatment. Report. Finnish Forest Research Institute. 4 pp. Not GLP. Unpublished.	Y	VRA
IIB 4.1.1	Vainio, E. Lipponen, K. Hantula, J.	2001	Persistence of a biological strain of *Phlebiopsis gigantea* in conifer stumps and its effects on within-species genetic diversity. For. Path. Vol. 31, pp. 285-295. Not GLP. Published.	N	
IIB 4.1.1	Korhonen, K.	2003a	Identification of fungal isolates from the biopreparates 1984, 1985 and 1986, made by Verdera Oy for treating conifer stumps against *Heterobasidion.* Report. Finnish Forest Research Institute. 2 pp. Not GLP. Unpublished.	Y	VRA
IIB 4.1.1	Holmer, L. Stenlid, J.	2003	New isolates of *Phlebiopsis gigantea*; methods and results. Report. Swedish University of Agricultural Sciences. 9 pp. Not GLP. Unpublished.	Y	VRA
IIB 4.1.1 IIB 4.1.2 IIB 4.1.4 IIB 4.1.5	Thorpe, K.	2005a	SOP: Method for selection of isolates for PG Suspension. Forest Research, UK. Not. GLP. Unpublished.	Y	FOC
IIB 4.1.1	Webber, J., Thorpe, K.	2003	Potential for biological control of *Heterobasidion annosum* in the UK using Rotstop. In: Laflamme *et al.* (eds.). Root and butt rots of forest trees. Proc. 10th Int. conf. on root and butt rots.Quebec City, Canada, 2001, pp. 221 – 225. Not GLP. Published.	N	FOC
IIB 4.1.2 IIB 4.1.4	Seiskari, P.	2005a	SOP 144-1. Maintenance and subculturing of microbial strains used in Rotstop production. Verdera Oy. 3pp. Not GLP. Unpublished.	Y	VRA
IIB 4.1.5	Seiskari ,P.	2004	SOP 101-1. Viability determination of biological pesticides. Verdera Oy, 4 pp. Not GLP. Unpublished.	Y	VRA

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
IIB 4.1.5 IIB 4.1.6	Seiskari, P.	2005b	SOP 120-2 Viability determination of biological pesticides using MPN-method. Verdera Oy. 4 pp. Not GLP. Unpublished.	Y	VRA
IIB 4.1.5	Seiskari, P.	2005h	SOP 143-1. Quality control of Rotstop production. Verdera Oy. 3 pp. Not GLP. Unpublished. Confidential information (Document C)	Y	VRA
IIB 4.1.2	Hallaksela, A-M., Korhonen, K.	1992b	Isolation of *P. gigantea* from a tree stump or log. Finnish Forest Research Institute. 1 p. Not GLP. Unpublished.	N	METLA
IIB 4.1.2	Seiskari, P.	2005c	SOP 147-1. Rotstop test on agar plates. Verdera Oy. 4 pp. Not GLP. Unpublished.	Y	VRA
IIB 4.1.2	Korhonen, K.	2003b	Simulated stump treatment experiments for monitoring the efficacy of *Phlebiopsis gigantea* against *Heterobasidion.* In: Laflamme *et al.* (eds.). Root and butt rots of forest trees. Proc. 10th Int. Conf. on Root and Butt Rots. Quebec City, Canada, 2001, pp 206-210. Not GLP. Not GLP. Published	N	
IIB 4.1.6	Seiskari, P.	2005d	SOP 163-3. Quality control in Rotstop production facilities. Verdera Oy. 1 p. Not GLP. Unpublished.	Y	VRA
IIB 4.1.6	Lahdenperä, M-L.	2005	SOP 165-1. Method for surveying viable microbes in the indoor air in the production facilities of Rotstop. Verdera Oy. 1 p. Not GLP. Unpublished.	Y	VRA
IIB 4.1.7	Seiskari, P.	2005e	Analysis of pathogenic contaminants in Rotstop. Report 159-1. Verdera Oy. 1 p. + 4 appendices. GLP Yes. Unpublished.	Y	VRA
IIB 4.1.8	Seiskari, P.	2005f	SOP 161-1. Storage stability of biological control agents. Verdera Oy. 1 p. Not GLP. Unpublished.	Y	VRA
IIB 4.1.8	Seiskari, P.	2005j	Storage stability of Rotstop. Report-149-1. Verdera Oy. 2 pp. Not GLP. Unpublished.	Y	VRA
Annex III Data and Information					
IIIB 5.1.4	Seiskari, P.	2005f	SOP 161-1. Storage stability of biological control agents. Verdera Oy. 1 p. Not GLP. Unpublished.	Y	VRA

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y/N	Owner **
IIIB 5.1.4	Pulkkanen, H	1995	Effect of temperature changes during storage and transportation on shelf-life of Rotstop. Memorandum 95093-Esp-Me. Kemira Agro Oy. 3 pp. Not GLP. Unpublished.	Y	VRA
IIIB 5.1.4	Woolley, A.J., Mullee, D.M.	2004a	Determination of accelerated storage stability. SPL Project number: 1841/005. SafePharm Laboratories. 27 pp. GLP Yes. Unpublished.	Y	VRA
IIIB 5.1.4	Woolley, A.J., Mullee, D.M.	2004b	Determination of long-term storage stability. SPL Project number: 1841/006. SafePharm Laboratories. 27 pp. GLP Yes. Unpublished.	Y	VRA
IIIB 5.1.4	Seiskari, P.	2002	Sedimentation test with Rotstop preparations made of Swedish *Phlebiopsis* strains. Test report. Verdera Oy. Not GLP. Unpublished.	Y	VRA
IIIB 5.1.4	Seiskari, P.	2005k	Tap density of Rotstop. Report-150-1. Verdera Oy. 1 p. Not GLP. Unpublished.	Y	VRA
IIIB 5	Briggs, L.H., Cambie, R.C., Dean, I.C., Dromgoole, S.H., Fergus, B.J., Ingram, K.G., Lewis, K.G., Small, C.W., Thomas, R. & Walker, D.A.	1975	Chemistry of fungi 10. Metabolites of some fungal species. N. Z. J. Sci. Vol. 18, pp. 565 – 576. Not GLP. Published.	N	
IIIB 5	Holdenrieder, O., Greig, B.J.W.	1998	Biological methods of control. In: Woodward *et al*. (eds). *Heterobasidion annosum*. Biology, Ecology, Impact and Control. CAB International, UK, pp. 235 – 258. Not GLP. Published.	N	
IIIB 5	Ikediugwu, F.E.O., Dennis, C., Webster, J.	1970	Hyphal interference by *Peniophora gigantea* and *Heterobasidion annosum*. Trans. Br. Mycol. Soc., Vol. 54 (2), pp. 307 – 309. Not GLP. Published.	N	
	Capretti, P., Mugnai, L.	1989	*In vitro* test of antagonism against *Heterobasidion annosum* (Fr.) Bref. Phytopath. Medit. Vol. 28, pp. 155 – 157. Not GLP. Published.	N	

**: Protection for 5 years claimed from date of decision concerning listing in Annex I - the study report has not been submitted any of the Member States in support of an application for authorization, or (though the study report has*

been submitted) has not been used any of the Member States as the basis for decision on the initial authorization, or to maintain a given authorization, of a plant protection product before the date of submission of the dossier to Rapporteur Member State.

***: Owners' code identifications and names:* VRA – Verdera; FOC – Forestry Commission; METLA - Finnish Forest Research Institute