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Gene expression analyses reveal a relationship between conidiation and cerato-platanin in homokaryotic and heterokaryotic strains of the fungal plant pathogen *Heterobasidion irregulare*

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Abstract

The Basidiomycete *Heterobasidion irregulare* was recently sequenced and three cerato-platanin encoding genes were found in its genome (*HiCPs*). Cerato-platanin family proteins (CPPs) are produced by both plant pathogenic and non-pathogenic fungi, and can act both as virulence factors and elicitors of defence responses. In fungal life these proteins seem to play a dual role, in the fungal cell wall and in the fungus-plant interaction, but most data available to date on CPPs derive from studies performed on Ascomycetes. In the present study, we investigated the expression of *HiCPs* in three homokaryotic isolates and two heterokaryotic isolates of the forest pathogen *H. irregulare*. Transcription of *HiCPs* was analysed both at the edge and at the centre of the fungal colony and compared between homokaryon and heterokaryon. Results showed that only *HiCP1* and *HiCP2* are likely to be translated in *H. irregulare* and that, under the tested conditions, *HiCP1* is by far the gene with the highest transcript abundance among *HiCPs*. *HiCP1* did not show any preferential expression in different sections of the fungal colony, while *HiCP2* was significantly more expressed at the colony centre, thus suggesting a link with the production of conidia. The level of expression of *HiCPs* in heterokaryons was generally comparable to that of one or both the parental homokaryons, irrespective of the colony section, thus demonstrating that *HiCPs* are not transcriptionally influenced by the heterokaryotic stage.

Keywords: MAMP, PAMP, snodprot, eliciting plant response, small protein, expansins

Introduction

Heterobasidion irregulare (Underw.) Garbel. & Otrosina is a Basidiomycete included in the species complex of *H. annosum* (Fr.) Bref. *sensu lato* (Garbelotto and Gonthier 2013). *H. irregulare* is a forest pathogen which, in North America, attacks pine (*Pinus* spp.), juniper (*Juniperus* spp.), and incense cedar (*Calocedrus decurrens*) (Garbelotto and Gonthier 2013). In Italy, where it was introduced during World War II (Garbelotto et al. 2013), the fungus has become invasive in Italian stone pine (*Pinus pinea*) stands (Gonthier et al. 2007). In the invasion area in Italy, *H. irregulare* is significantly more widespread than its congener *H. annosum sensu stricto* (Gonthier et al. 2007, 2014). The two species hybridise, and it was reported that a massive allele introgression mostly occurs unilaterally from the native species to the invasive one (Gonthier and Garbelotto 2011).

In nature, homokaryotic haploid mycelia are responsible for primary infections occurring on the surface of fresh stumps or wounds on the trees, while secondary mycelia, consisting of a mosaic of haploid and heterokaryotic mycelia, may be responsible for the infections occurring from stump to tree or from tree to tree, through root contacts or grafts (Stenlid and Rayner 1991; Garbelotto and Gonthier 2013).

H. irregulare is a wood decay agent that can switch from a saprotrophic lifestyle on stumps to a necrotrophic parasitism on living trees (Olson et al. 2012), and is able to degrade all components of wood, including lignin and cellulose (Yakovlev et al. 2012, 2013; Raffaello et al. 2013). Recently, with the aim of investigating the molecular bases of the two trophic strategies, the genome of *H. irregulare* has been sequenced and, for the first time, the presence of genes encoding cerato-platanin family proteins (CPPs) has been reported (Olson et al. 2012). In that study, the authors included *CPP* genes among the pathogenicity factors of the fungus.

CPPs are an enigmatic family of proteins found exclusively in filamentous fungi that seem to actually play a role in virulence for some pathogens (Jeong et al. 2007; Frías et al. 2011). However, most studies have investigated the ability of CPPs to act as elicitors of defence responses when separately applied to plants (Djonović et al. 2006; Yang et al. 2009; Frías et al. 2013; Baccelli et al. 2014a). Fungi abundantly secrete CPPs into their culture media, but some studies have also localised these proteins within the fungal cell wall (Gaderer et al. 2014; Pazzagli et al. 2014). As recently demonstrated, CPPs are not hydrophobin-like proteins, because they have different biochemical and structural properties (de Oliveira et al. 2011; de O. Barsottini et al. 2013; Frischmann et al. 2013). CPPs are instead partially similar to proteins named expansins, which in plants mediate cell wall loosening and are involved in various processes like growth (Sampedro and Cosgrove 2005; Baccelli et al. 2014b). On the basis of the results obtained up to date, it seems plausible that, in fungal life, CPPs may act in an expansin-like manner and cause the loosening of both fungal and plant cell walls (Baccelli 2015). Ascomycetes usually have one or two *CPP* encoding genes and most information available to date on CPPs comes from these fungi (Chen et al. 2013; Gaderer et al. 2014). Basidiomycetes may have up to twelve *CPP* genes, but only those from *Moniliophthora perniciosa* have been studied up to date (Chen et al. 2013; de O. Barsottini et al. 2013).

In the present study, we aimed at investigating the transcriptional regulation of *CPPs* in the Basidiomycete *H. irregulare*, which harbours three *CPP* encoding genes in its genome (Olson et al. 2012). We analysed and compared the transcription of *CPPs* in five isolates, three homokaryotic isolates and two heterokaryotic isolates. Homokaryotic and heterokaryotic mycelia not only play different roles in the infectious process, but also differ in their biology: clamp connections are formed in the heterokaryotic mycelia. As CPPs have been found localised in the fungal cell wall and may act as expansins, we hypothesised that the expression of *CPP* genes could be influenced in the heterokaryotic mycelia. In addition, we tested whether *CPP* genes were differently transcribed within the fungal colony in relation to other morphological or physiological features.

Materials and methods

Fungal strains and culture conditions

Three homokaryotic haploid isolates and two heterokaryotic isolates of *H. irregulare* were used in this study. Homokaryotic isolates 142EF, 53OA and 89EG were obtained from spores landed on woody spore traps exposed in the Circeo National Park (Sabaudia, Italy), Gallinara pinewood (Anzio, Italy), and La Campana pinewood (Nettuno, Italy), respectively, and were collected and isolated in pure culture as previously described (Gonthier and Garbelotto 2011). The purity of the isolates (over 95% assignment to *H. irregulare*) was assessed in a study based on the characterisation of over 500 AFLP loci (Gonthier and Garbelotto 2011). The two heterokaryotic isolates 142x53OA and 142x89EG were obtained in the laboratory by mating the isolates 142EF with 53OA, and 142EF with 89EG, respectively. Inocula of the clampless parental homokaryotic isolates were placed about 1 cm apart in the middle of a 9-cm diameter Petri dish filled with 1.5% malt extract agar (MEA) (Difco, Detroit, MI). After 3 weeks, a small piece of mycelium was taken

from the zone of contact of the two isolates and transferred into a new Petri dish, resulting in a heterokaryotic isolate as assessed under 200x magnification for the presence of clamp connections.
The isolates were grown and maintained in 9-cm diameter Petri dishes on MEA at 23 °C, in the dark. In order to facilitate removal of mycelium for the subsequent DNA and RNA extractions, the isolates were grown on a cellophane disc placed on the surface of the culture medium. Cellophane discs were sterilised in autoclave at 121 °C for 15 min.

DNA extraction and sequencing of cerato-platanin encoding genes

DNA was obtained from the homokaryotic haploid isolates by grinding 30–50 mg of mycelium in 200 µl of Hexadecyltrimethylammonium bromide (CTAB) lysis buffer (NaCl 1.4 M; EDTA 20 mM; Tris-HCl 100 mM, pH 8.0; CTAB 3% w/v; 2-Mercaptoethanol 0.2% v/v). DNA was then extracted with isoamyl alcohol-chloroform and precipitated with isopropyl alcohol.
The coding regions of the *CPP* genes were amplified with PCR by using primers designed on the basis of the sequences and annotations present in JGI MycoCosm (Fungal Genomic Resource, Joint Genome Institute) for the sequenced strain TC32-1 of *H. irregulare* (Olson et al. 2012). Both forward and reverse primers were designed on the untranslated regions (UTRs) 5' and 3'.
The following primers were designed and used (HiCP stands for *H. irregulare* cerato-platanin): *HiCP1* Forward (For) 5'-GTGCTCTCATCTCTGTCGTCC, Reverse (Rev) 5'-TCCAAAGCGTAACGATCTTCCT; *HiCP2* For 5'-CACCAATTCATTGCATTTACACACT, Rev 5'-AGGCTCGTGCATACATGTGAA; *HiCP3* For 5'-CTCACACTCGACTAGCGCAT, Rev 5'-TGGAACACTTGTATCTCACCAT. In addition, *HiCP2* sequences were further extended at the 5'-end by using the primers For 5'-GGACAGCCATATCTTCCGACACC and Rev 5'-GGGACGTAGATGGTTTGGACC.
All the reactions were carried out with 2.5 mM $MgCl_2$, 200 µM dNTPs, 400 nM primer (each), 1X Reaction Buffer, and 0.05 U μl^{-1} EuroTaq DNA polymerase (Euro Clone, Italy), at an annealing temperature of 57 °C for 28–30 cycles.
The reaction products were purified from electrophoresis gel with Wizard SV Gel and PCR Clean-Up System (Promega Italia Srl, Italy) and sequenced by Eurofins MWG Operon/Carlo Erba Reagents Srl, Italy. In order to avoid the presence of amplification mistakes within the sequence, both amplification reactions and sequencing were repeated.
The sequences obtained were analysed with on-line tools such as ClustalW, ExPASy and BLAST software, and finally deposited in GenBank under the accession numbers LN626599–LN626607. Signal peptide predictions were performed with PrediSi.

Growth rates

The radial growth rate of each isolate was determined on MEA in 9-cm Petri dishes, at 23 °C, in the dark, by growing the fungus on a cellophane disc for the subsequent RNA extraction. The Petri dishes were inoculated in the centre with an 8-mm diameter agar plug obtained from the edge of actively growing fungal cultures. The radial growth was measured daily along two perpendicular lines during 6 days. Five replicates were grown per each isolate and the experiment was repeated three times over a period of 3 months.

Microscopic analysis

Microscopical observations of conidial presence were performed both from the edge (last 24-h growth) and from the centre of the colony (48–144 h), after differentiating mycelium sections as described in the following paragraph. The production of conidia and conidiophores was determined by examining 5 field of view (FOV) at 200x magnification on three experimental replicates obtained as described above. The production of conidiophores was expressed as number per FOV, whereas the production of conidia was expressed as +, ++, or +++ depending on the relative abundance of dispersed conidia found in the samples.

RNA extraction and transcription analysis

Mycelium of each isolate was collected from the cellophane layer of a randomly selected culture by respecting the following scheme: from the edge of the colony it was collected the mycelium grown in the last 24 h (i.e. 0–24 h old); the mycelium 24–48 h old was left on the plate and considered as an intermediate zone; the mycelium in the centre of the colony was collected from the intermediate zone up to the edge of the agar plug (i.e. 48–144 h old). The

intermediate zone was not subjected to further analysis with the aim of enhancing possible differences in the level of expression between the mycelium sections.

Total RNA was isolated from mycelium with RNeasy Plant Mini Kit (Qiagen, CA) by using RLT buffer. RNA was treated with DNase by using Amplification Grade DNase I (Sigma-Aldrich, MO) and reverse-transcribed (400 ng per sample) into cDNA with iScript cDNA synthesis kit (BioRad, USA).

Real-time qPCR reactions (20 µl) were carried out with 10 ng of cDNA, 250 nM primers, and 1x Fast SYBR Green Master Mix (Applied Biosystems, CA) following the manufacturer's instructions. PCRs were run in a StepOne real-time PCR System (Applied Biosystems) by using the recommended thermal-cycling conditions (hold 95 °C, 20 s; 40 cycles 95 °C, 3 s; 60 °C, 30 s). Gene specific primers for *HiCP1*, *HiCP2* and *HiCP3* were designed with Primer Express Software 3.0 (Applied Biosystems) so that the allelic variants resulted from the previous sequencing could not be distinguished. The following primers were designed and used: *HiCP1* For 5'-CACGAACGGCCTCATCAAC, Rev 5'-GACGTTCGGGAAAGACGGTAA; *HiCP2* For 5'-CCCGACCTTCAGCGATCTAC, Rev 5'-ACCCGACGGCGAAAGC; HiCP3 For 5'-CAGTTCTACGCCAAGTGCCTACT, Rev 5'-GACGTGCCGCTGGGATAA.

Relative gene expression values ($2^{-\Delta\Delta Ct}$) were calculated by using *18S* rRNA gene as the endogenous reference gene following the calculation described in ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems). *18S* primers were designed by using the GenBank sequence AF026576 (primers For 5'-TGGTGCATGGCCGTTCTT, Rev 5'-AGCAGGTTAAGGTCTCGTTCGT). *18S* was used as the reference gene after confirmation of its transcriptional stability across isolates and mycelium sections.

Before the quantification, a validation experiment was performed to ensure that the amplification efficiencies of the target genes and the reference gene were comparable.

Statistical analysis

Relative gene expression data ($2^{-\Delta\Delta Ct}$) were analysed with one-sample *t* test (centre vs. edge=1) or with one-way ANOVA with Tukey-Kramer post test (heterokaryon vs. parental homokaryons; common parental 142EF=1). Growth rates and production of conidiophores were similarly analysed with one-way ANOVA with Tukey-Kramer post test at $P<0.05$. All the statistical analyses were performed by using GraphPad InStat v. 3.05 (GraphPad Software, San Diego, CA).

Results

Sequencing of cerato-platanin encoding genes in Italian isolates of *Heterobasidion irregulare*

The length of *HiCP* sequences from the isolates 142EF, 53OA and 89EG was 545 bp for *HiCP1*, 1032–1035 bp for *HiCP2* and 651 bp for *HiCP3*. *HiCP1* coded for a protein of 138 amino acids, with a putative N-terminal signal peptide of 19 amino acids (Fig. 1), and showed the canonical domain structure of CPPs (Chen et al. 2013). In addition, by sequencing *HiCP1* from DNA and cDNA, we confirmed the presence of a 65-bp intron located in the 3'UTR (Online Resource, Fig. S1). *HiCP2* coded for a protein of 259–260 amino acids, with a putative signal peptide of 19 amino acids, and was composed of an N-terminal region with many repetitions with no apparent similarity to known proteins, and a C-terminal region which showed similarities with the CPP domain. Finally, *HiCP3* seemed to encode for a truncated version of *HiCP2* of 105 amino acids, without a signal peptide, and was apparently contained in a putative longer transcript of about 4 kb (data not shown).

By comparing the sequences obtained in this study with those available from the American strain TC32-1, we found that the deduced amino acid sequences of *HiCP1* and *HiCP3* were highly conserved (Fig. 1). In fact, all the three homokaryotic Italian isolates and TC32-1 showed the same amino acid sequence. However, we found five putative single nucleotide polymorphisms (SNPs) in the nucleotide sequence of *HiCP1*, two in the coding region and three within the intron (Online Resource, Fig. S1). *HiCP2* showed instead more polymorphisms, both at the level of gene and protein sequences, including a deletion in the Italian isolate 53OA (Fig. 1 and Online Resource, Fig. S2). *HiCP3* showed only one putative SNP (Online Resource, Fig. S3).

Transcription analysis of cerato-platanin genes

Based on a general analysis of the qPCR data, *HiCP1* always turned out to be the *CPP* gene with the highest transcript abundance, with a level of transcription at least 300 fold higher than *HiCP2* or *HiCP3*; *HiCP2* was generally more transcribed than *HiCP3* (Online Resource, Table S1).

When mycelium collected from the centre of the colony (48–144 h old) was compared to mycelium collected from actively growing hyphae (0–24 h old, edge of the colony), no significant difference in the relative level of transcription was found for *HiCP1* (Fig. 2). Interestingly, when *HiCP2* was analysed, it was significantly more expressed at the centre of the colony, and this was true in all isolates. The transcription level of *HiCP3* was not significantly different in the two tested colony sections, although the isolate 142EF showed a high transcription level in the centre of the colony.

The heterokaryon 142EFx53OA showed, for *HiCP1*, the same level of transcription as the parental homokaryons in both tested colony sections (Fig. 3A); for *HiCP2* and *HiCP3* it showed instead differences compared to the homokaryon 142EF, either in the colony centre (*HiCP2*) or in both the sections (*HiCP3*), but no significant differences were observed between the heterokaryon and the other parental homokaryon 53OA (Fig. 3A). Concerning the combination 142EF with 89EG (Fig. 3B), although *HiCP1* was slightly more expressed in the centre of the colony at the heterokaryotic stage, in all the other cases the level of expression of the *CPP* genes in the heterokaryon was comparable to that of one or both the parental homokaryotic isolates.

Morphological analysis of the isolates

Growth rate and production of conidia and conidiophores were analysed in order to find possible relationships with the expression pattern of the *CPP* genes. Neither conidia nor conidiophores were observed at the edge of the colony, i.e. the younger part of the mycelium; on the contrary, all the strains abundantly produced conidia and conidiophores at the centre. The heterokaryotic isolates released less conidia than the parental homokaryotic isolates, but the isolates did not statistically differ in the production of conidiophores (Table 1).

The average radial growth rates ranged from 4.5 mm day^{-1} of the isolate 142EF to 6.2 mm day^{-1} of the isolate 89EG (Fig. 4). With the only exception represented by this comparison, differences in the growth rate between isolates were not significant.

Discussion

CPPs have been found in more than 50 fungal genomes, and Basidiomycetes show both the higher sequence diversity and number of homologs (Chen et al. 2013). However, the current knowledge concerning CPPs almost exclusively derives from studies performed on Ascomycetes. The hemibiotroph *Moniliophthora perniciosa*, the causal agent of witches' broom disease in cacao, is the only Basidiomycete where CPPs have been studied so far (de O. Barsottini et al. 2013). In the present study, we investigated the regulation of *CPPs* in a necrotrophic Basidiomycete, the conifer root rot pathogen *H. irregulare*, and obtained for the first time data on how these genes are transcribed within the fungal colony at the homokaryotic and heterokaryotic stage.

Based on the sequencing and annotation of the North American *H. irregulare* strain TC32-1, we identified three *CPP* encoding genes: *CerPla1*, *CerPla2* and *CerPla3* (Olson et al. 2012). However, in order to follow the current denomination of CPPs and to differentiate them clearly from *cerato-platanin* from *C. platani*, here we propose to rename these genes as *HiCP1* (*H. irregulare cerato-platanin 1*), *HiCP2* and *HiCP3*.

The sequence analysis showed that *H. irregulare* possesses three highly different CPPs, with a length of 138 amino acids (HiCP1), 259–260 amino acids (HiCP2) and 105 amino acids (HiCP3). Thus, this analysis firstly allowed correction of the previous sequence annotations: six terminal amino acids were added to HiCP2; a putative start codon was identified in HiCP3.

HiCP1 was the only HiCP with the canonical structure of CPPs, i.e. typical length with signal peptide (Chen et al. 2013), and was also the gene showing the highest transcript abundance. *HiCP2* and *HiCP3* were both transcribed as well, but to a lower extent. Nevertheless, the translation into a protein of *HiCP3* seemed unlikely for several reasons: it appeared as a truncated version of *HiCP2*, the localisation of the start codon was accordingly uncertain, a signal peptide could not be found, and the length of the protein sequence was the shortest ever reported so far for CPPs (Chen et al. 2013).

The transcriptional study was performed on five isolates which were very similar in their growth rate. Thus, we did not attempt to compare the transcription level of *HiCPs* between isolates to find correlations with their growth rate. We studied instead the transcription of *HiCPs* within each colony, by comparing the edge with the centre.

We found that *HiCP1* was transcribed by the isolates without significant differences between colony sections, while *HiCP2* showed a clear expression pattern: it was significantly more transcribed at the centre of the colony, and this was true in all isolates. The edge and the centre of the colony are two clearly different physiological and morphological zones: at the edge of the colony the fungus has actively growing hyphae lacking conidiophores and conidia, while the colony centre represents the older part with abundant conidia. Therefore, this result may suggest a link between *HiCP2*

and the production of conidia, according to the evidence of a functional diversification and specialisation of CPP homologs (de O. Barsottini et al. 2013; Frischmann et al. 2013). It is also interesting to note that, although CPPs do not possess the biochemical properties of hydrophobins (Frischmann et al. 2013), the gene induction during conidiation is reminiscent of hydrophobins (Dubey et al. 2014).

No relative increase in the transcription level of *HiCPs* was found in the heterokaryotic mycelia. The level of expression in heterokaryons was generally comparable to that of one or both the parental homokaryons, irrespective of the colony section, thus demonstrating that *HiCPs* are not transcriptionally influenced by the heterokaryotic stage.

In conclusion, this study has shown for the first time that *CPP* genes are differently transcribed within the fungal colony, and this occurs in both homokaryotic and heterokaryotic mycelia. However, their expression level is not altered by heterokaryosis. Our findings suggest that only *HiCP1* and *HiCP2* are likely to play a role in the biology of *H. irregulare*, with *HiCP1* having probably the major role. In fact, *HiCP1* showed the highest transcript abundance in all isolates and it did not show preferential expression in different sections of the colony. Further studies will help to understand whether *HiCP1* can play a role in both hyphal elongation and the production of conidia, by acting as expansin-like protein in the cell wall as similarly suggested for *CP* from *C. platani* (Baccelli et al. 2012; Baccelli 2015), and whether *HiCP2* actually has a preferential role during the formation of conidia, similarly to the *CPP* gene *epl2* from *Trichoderma atroviride* (Frischmann et al. 2013).

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Conflict of Interest

The authors declare that they have no conflict of interest

References

Baccelli I, Comparini C, Bettini PP, Martellini F, Ruocco M, Pazzagli L, Bernardi R, Scala A (2012) The expression of the cerato-platanin gene is related to hyphal growth and chlamydospores formation in *Ceratocystis platani*. FEMS Microbiol Lett 327:155–163

Baccelli I, Lombardi L, Luti S, Bernardi R, Picciarelli P, Scala A, Pazzagli L (2014a) Cerato-platanin induces resistance in Arabidopsis leaves through stomatal perception, overexpression of salicylic acid- and ethylene-signalling genes and camalexin biosynthesis. PLoS One 9:e100959

Baccelli I, Luti S, Bernardi R, Scala A, Pazzagli L (2014b) Cerato-platanin shows expansin-like activity on cellulosic materials. Appl Microbiol Biotechnol 98:175–184

Baccelli I (2015) Cerato-platanin family proteins: one function for multiple biological roles?. Front Plant Sci 5:769

Chen H, Kovalchuk A, Keriö S, Asiegbu FO (2013) Distribution and bioinformatic analysis of the cerato-platanin protein family in Dikarya. Mycologia 105:1479–1488

de O Barsottini MR, de Oliveira JF, Adamoski D, Teixeira PJ, do Prado PF, Tiezzi HO, Sforça ML, Cassago A, Portugal RV, de Oliveira PS, de M Zeri AC, Dias SM, Pereira GA, Ambrosio AL (2013) Functional diversification of cerato-platanins in *Moniliophthora perniciosa* as seen by differential expression and protein function specialization. Mol Plant Microbe Interact 26:1281–1293

de Oliveira AL, Gallo M, Pazzagli L, Benedetti CE, Cappugi G, Scala A, Pantera B, Spisni A, Pertinhez TA, Cicero DO (2011) The structure of the elicitor Cerato-platanin (CP), the first member of the CP fungal protein family, reveals a double ψβ-barrel fold and carbohydrate binding. J Biol Chem 286:17560–17568

Djonović S, Pozo MJ, Dangott LJ, Howell CR, Kenerley CM (2006) Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Mol Plant Microbe Interact 19:838–853

Dubey MK, Jensen DF, Karlsson M (2014) Hydrophobins are required for conidial hydrophobicity and plant root colonization in the fungal biocontrol agent *Clonostachys rosea*. BMC Microbiol 14:18

Frías M, Brito N, González C (2013) The *Botrytis cinerea* cerato-platanin BcSpl1 is a potent inducer of systemic acquired resistance (SAR) in tobacco and generates a wave of salicylic acid expanding from the site of application. Mol Plant Pathol 14:191–196

Frías M, González C, Brito N (2011) BcSpl1, a cerato-platanin family protein, contributes to *Botrytis cinerea* virulence and elicits the hypersensitive response in the host. New Phytol 192:483–495
Frischmann A, Neudl S, Gaderer R, Bonazza K, Zach S, Gruber S, Spadiut O, Friedbacher G, Grothe H, Seidl-Seiboth V (2013) Self-assembly at air/water interfaces and carbohydrate binding properties of the small secreted protein EPL1 from the fungus *Trichoderma atroviride*. J Biol Chem 288:4278–4287
Gaderer R, Bonazza K, Seidl-Seiboth V (2014) Cerato-platanins: a fungal protein family with intriguing properties and application potential. Appl Microbiol Biotechnol 98:4795–4803
Garbelotto M, Gonthier P (2013) Biology, epidemiology, and control of *Heterobasidion* species worldwide. Annu Rev Phytopathol 51:39–59
Garbelotto M, Guglielmo F, Mascheretti S, Croucher PJ, Gonthier P (2013) Population genetic analyses provide insights on the introduction pathway and spread patterns of the North American forest pathogen *Heterobasidion irregulare* in Italy. Mol Ecol 22:4855–4869
Gonthier P, Anselmi N, Capretti P, Bussotti F, Feducci M, Giordano L, Honorati T, Lione G, Luchi N, Michelozzi M, Paparatti B, Sillo F, Vettraino AM, Garbelotto M (2014) An integrated approach to control the introduced forest pathogen *Heterobasidion irregulare* in Europe. Forestry 87:471–481
Gonthier P, Garbelotto M (2011) Amplified fragment length polymorphism and sequence analyses reveal massive gene introgression from the European fungal pathogen *Heterobasidion annosum* into its introduced congener *H. irregulare*. Mol Ecol 20:2756–2770
Gonthier P, Nicolotti G, Linzer R, Guglielmo F, Garbelotto M (2007) Invasion of European pine stands by a North American forest pathogen and its hybridization with a native interfertile taxon. Mol Ecol 16:1389–1400
Jeong JS, Mitchell TK, Dean RA (2007) The *Magnaporthe grisea* snodprot1 homolog, MSP1, is required for virulence. FEMS Microbiol Lett 273:157–165
Olson A, Aerts A, Asiegbu F, Belbahri L, Bouzid O, Broberg A, Canbäck B, Coutinho PM, Cullen D, Dalman K, Deflorio G, van Diepen LT, Dunand C, Duplessis S, Durling M, Gonthier P, Grimwood J, Fossdal CG, Hansson D, Henrissat B, Hietala A, Himmelstrand K, Hoffmeister D, Högberg N, James TY, Karlsson M, Kohler A, Kües U, Lee YH, Lin YC, Lind M, Lindquist E, Lombard V, Lucas S, Lundén K, Morin E, Murat C, Park J, Raffaello T, Rouzé P, Salamov A, Schmutz J, Solheim H, Ståhlberg J, Vélëz H, de Vries RP, Wiebenga A, Woodward S, Yakovlev I, Garbelotto M, Martin F, Grigoriev IV, Stenlid J (2012) Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. New Phytol 194:1001–1013
Pazzagli L, Seidl-Seiboth V, Barsottini M, Vargas WA, Scala A, Mukherjee PK (2014) Cerato-platanins: elicitors and effectors. Plant Sci 228:79–87
Raffaello T, Chen H, Kohler A, Asiegbu FO (2013) Transcriptomic profiles of *Heterobasidion annosum* under abiotic stresses and during saprotrophic growth in bark, sapwood and heartwood. Environ Microbiol 16:1654–1667
Sampedro J, Cosgrove DJ (2005) The expansin superfamily. Genome Biol 6:242
Stenlid J, Rayner ADM (1991) Patterns of nuclear migration and heterokaryosis in pairings between sibling homokaryons of *Heterobasidion annosum*. Mycol Res 95:1275–1283
Yakovlev I, Vaaje-Kolstad G, Hietala AM, Stefańczyk E, Solheim H, Fossdal CG (2012) Substrate-specific transcription of the enigmatic GH61 family of the pathogenic white-rot fungus *Heterobasidion irregulare* during growth on lignocellulose. Appl Microbiol Biotechnol 95:979–990
Yakovlev IA, Hietala AM, Courty PE, Lundell T, Solheim H, Fossdal CG (2013) Genes associated with lignin degradation in the polyphagous white-rot pathogen *Heterobasidion irregulare* show substrate-specific regulation. Fungal Genet Biol 56:17–24
Yang Y, Zhang H, Li G, Li W, Wang X, Song F (2009) Ectopic expression of MgSM1, a Cerato-platanin family protein from *Magnaporthe grisea*, confers broad-spectrum disease resistance in Arabidopsis. Plant Biotechnol J 7:763–777

Supplementary Material

Fig. S1 ClustalW alignment of *HiCP1* nucleotide sequences
Fig. S2 ClustalW alignment of *HiCP2* nucleotide sequences
Fig. S3 ClustalW alignment of *HiCP3* nucleotide sequences
Table S1 Average ΔCt values

Table 1 Production of conidia and conidiophores in different zones of mycelium

	Centre of the colony[a]		Edge of the colony[b]	
Isolate	Conidiophores[c]	Conidia[d]	Conidiophores	Conidia
53OA	6.6±5.9 (a)	+++	n.o.	n.o.
89EG	7.2±4.3 (a)	+++	n.o.	n.o.
142EF	3.9±0.5 (a)	+++	n.o.	n.o.
142EFx53OA	3.5±1.8 (a)	++	n.o.	n.o.
142EFx89EG	4.4±1.2 (a)	++	n.o.	n.o.

[a] Mycelium 48–144 h old.
[b] Mycelium 0–24 h old.
[c] Conidiophores are expressed as average number ± SD per field of view (FOV) at 200x; n.o., not observed.
[d] Dispersed conidia are expressed as (++) or (+++) depending on the relative abundance in the strain samples; n.o., not observed.
Five FOVs per replicate were examined. Data represent three replicates. Statistical analysis was performed with one-way ANOVA with Tukey-Kramer post test at $P<0.05$.

Figure legends

Fig. 1 Amino acid alignment of *Heterobasidion irregulare* cerato-platanins (HiCPs). Deduced amino acid sequences were aligned with ClustalW. Italian homokaryotic isolates (142EF, 89EG, 53OA) were aligned with the American strain TC32-1. The annotations in MycoCosm for TC32-1 were reconsidered after BLASTX analysis by adding six C-terminal amino acids in HiCP2 and by identifying a putative start codon in HiCP3. The cerato-platanin domain is underlined. Conserved cysteines and signature sequences (CSD or CSN) of the family are highlighted (Chen et al. 2013). Boxes indicate the predicted signal peptide. Asterisks indicate invariable residues

Fig. 2 Relative expression of cerato-platanin encoding genes (*HiCPs*) within the fungal colony (centre vs. edge). The analysis was performed in five isolates of *Heterobasidion irregulare*, three homokaryons (142EF, 89EG, 53OA) and two heterokaryons (142EFx53OA, 142EFx89EG). Relative gene expression values were determined with real time RT-PCR by comparing mycelium collected from the centre of the colony (48–144 h old) to mycelium collected from the colony edge (0–24 h old), which was used as the calibrator in the analysis ($2^{-\Delta\Delta Ct}$ or fold change value=1). Average fold change values ± SD from three biological replicates are shown. Statistical analysis was performed with one-sample *t* test. Asterisk indicates significantly different at $P<0.05$

Fig. 3 Relative expression of cerato-platanin genes (*HiCPs*) in homokaryotic and heterokaryotic mycelia. The heterokaryons 142EFx53OA (a) and 142EFx89EG (b) were analysed in comparison to the respective parental homokaryons. The analysis was performed both at the colony centre and at the colony edge. Relative gene expression values were determined with real time RT-PCR by using the common parental 142EF as the calibrator in the analysis ($2^{-\Delta\Delta Ct}$ or fold change value=1). Average fold change values ± SD from three biological replicates are shown. Statistical analysis was performed with one-way ANOVA with Tukey-Kramer post test at $P<0.05$

Fig. 4 Radial growth rate of the isolates of *Heterobasidion irregulare* used in the present study. The isolates were grown on malt extract agar, at 23 °C, for 6 days. Five replicates were grown per each isolate and the experiment was repeated three times over a period of three months. Average data with SD are shown. Statistical analysis was performed with one-way ANOVA with Tukey-Kramer post test at $P<0.05$

1 Fig.1

HiCP1

```
TC32-1      MKFTFALASLAVLASTAFATDVRYDETYDNANEPLTDVACSDGTNGLINKGFSTLGSLPS
142EF       MKFTFALASLAVLASTAFATDVRYDETYDNANEPLTDVACSDGTNGLINKGFSTLGSLPS
89EG        MKFTFALASLAVLASTAFATDVRYDETYDNANEPLTDVACSDGTNGLINKGFSTLGSLPS
53OA        MKFTFALASLAVLASTAFATDVRYDETYDNANEPLTDVACSDGTNGLINKGFSTLGSLPS
            ************************************************************

TC32-1      FPNVAAVQAIAGWNSPSCGTCWEVTYNGRSVLVTGVDHAGDGINMSLEAMNTLTNNQGVA
142EF       FPNVAAVQAIAGWNSPSCGTCWEVTYNGRSVLVTGVDHAGDGINMSLEAMNTLTNNQGVA
89EG        FPNVAAVQAIAGWNSPSCGTCWEVTYNGRSVLVTGVDHAGDGINMSLEAMNTLTNNQGVA
53OA        FPNVAAVQAIAGWNSPSCGTCWEVTYNGRSVLVTGVDHAGDGINMSLEAMNTLTNNQGVA
            ************************************************************

TC32-1      LGTVSATVTQVAASQCGL
142EF       LGTVSATVTQVAASQCGL
89EG        LGTVSATVTQVAASQCGL
53OA        LGTVSATVTQVAASQCGL
            ******************
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HiCP2

```
TC32-1      MKFTASFIAVAALFHGTAAAPQDGGAPPTPNSPSGTSATSAVTWSKPSTSPVYSSTTSTY
142EF       MKFTASFIAVAALFHGTAAAPQDGGASPTPNSPSGTSATSAVTWSKPSTSPVYSSTTSTY
89EG        MKFTASFIAVAALFHGTAAAPQDGGASPTPNSPSGTSATSAVTWSKPSTSPVYSSTTSTY
53OA        MKFTASFIAVAALFHGTAAAPQDGGAPPTPNSPSGTSATSAVTWSKPSTSPVYSSTTSTY
            ***************************.********************************

TC32-1      SSTTSTTSPYTTTSATTTWYPSSTSSWYPPSTSSKPYPSSSSYPVPSSSHSYPPPPSGSA
142EF       SSTTSTTSPYTTTSATTTWYPSSTSSWYPPSTSSKPYPSSSSYPVPSSSHSYPPPPSGSA
89EG        SSTTSTTSPYTTTSATTTWYPSSTSSWYPPSTSSKPYPSSSSYPVPSSSHSYPPPPSGSA
53OA        SSTTSTTSPYTT-SATTTWYPSSTSSWYPPSTSSKPYPSSSSYPVPSSSHSYPPPPSGSA
            ************ ************************************************

TC32-1      TPPYPSNCPPNPNSNPLMMSLPLTYDNTYDNGSGSMNSVACSNGPKGLVERFPTFSDLPT
142EF       TPPYPSNCPPNPNSNPLMMSLPLTYDNTYDNGSGSMNSVACSNGPKGLVGRFPTFSDLPT
89EG        TPPYPSNCPPNPNSNPLMMSLPLTYDNTYDNGSGSMNSVACSNGPKGLVGRFPTFSDLPT
53OA        TPPYPSNCPPNPNSNPLMMSLPLTYDNTYDNGSGSMNSVACSNGPKGLVERFPTFSDLPT
            ********************************************* **********

TC32-1      FPYIGGAFAVGSWSSPNCGSCWSLTYPQTGVTIKLIAIDTSGVGFNAAQAAMDKLTGGKA
142EF       FPYIGGAFAVGSWSSPNCGSCWSLTYPQTGVTIKLIAIDTSGVGFNAAQAAMDKLTGGKA
89EG        FPYIGGAFAVGSWSSPNCGSCWSLTYPQTGVTIKLIAIDTSGVGFNAAQAAMDKLTGGKA
53OA        FPYIGGAFAVGSWSSPNCGSCWSLTYPQTGVTIKLIAIDTSGVGFNAAQAAMDKLTGGKA
            ************************************************************

TC32-1      NQLGRIEVNAYQLPASECKL
142EF       NQLGRIEVNAYQLPASECKL
89EG        NQLGRIEVNAYQLPASECKL
53OA        NQLGRIEVNAYQLPASECKL
            ********************
```

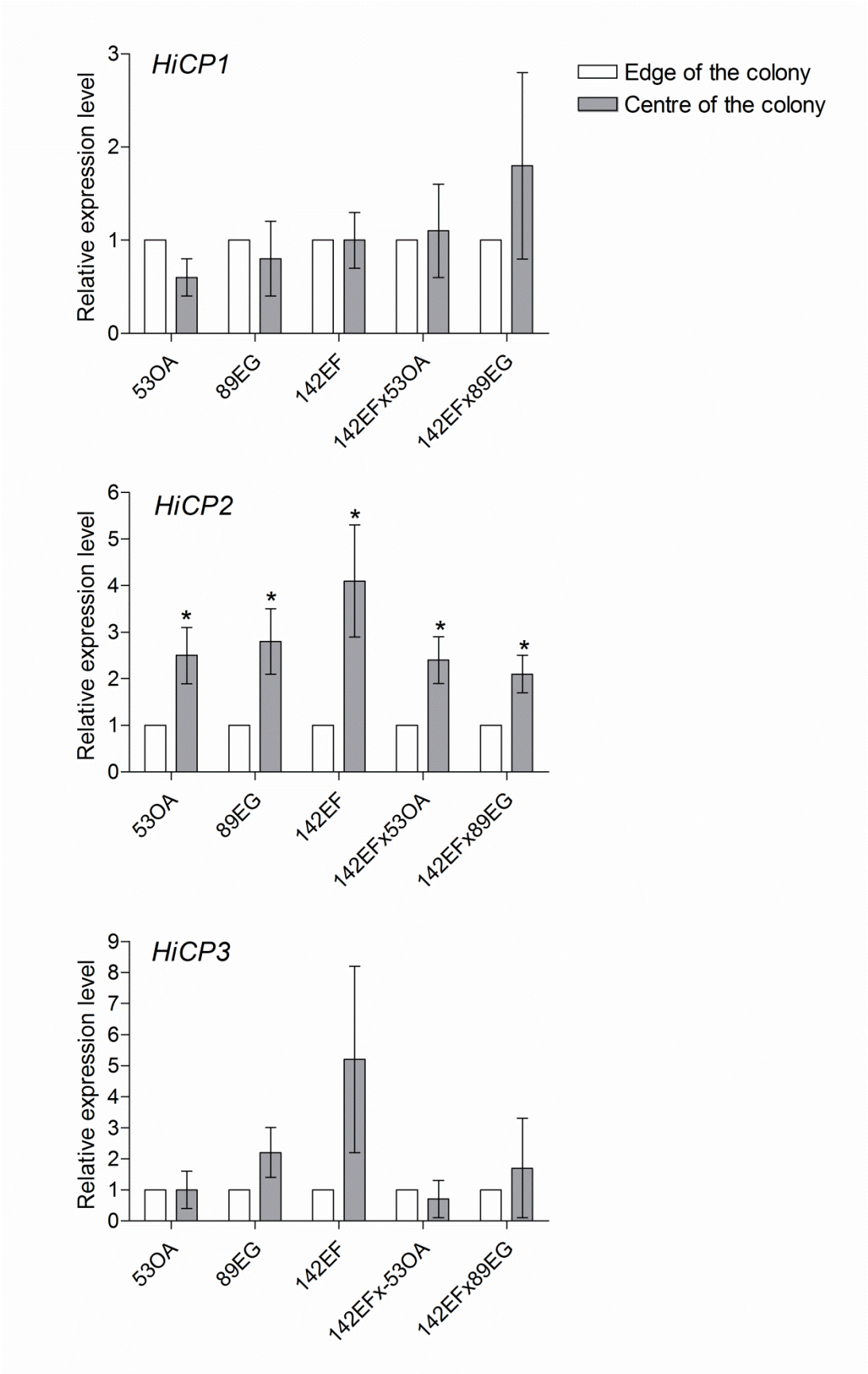
HiCP3

```
TC32-1      MNTAACSNGPHGLASKFPTFGDLPDYPYVGGVFAVSSWNSANCGTCWAVTYPETGVTINV
142EF       MNTAACSNGPHGLASKFPTFGDLPDYPYVGGVFAVSSWNSANCGTCWAVTYPETGVTINV
89EG        MNTAACSNGPHGLASKFPTFGDLPDYPYVGGVFAVSSWNSANCGTCWAVTYPETGVTINV
53OA        MNTAACSNGPHGLASKFPTFGDLPDYPYVGGVFAVSSWNSANCGTCWAVTYPETGVTINV
            ************************************************************

TC32-1      LAIDVASPGFNVAQAAMDKLTNGKATQLGKVEVNVEQVPTSACKL
142EF       LAIDVASPGFNVAQAAMDKLTNGKATQLGKVEVNVEQVPTSACKL
89EG        LAIDVASPGFNVAQAAMDKLTNGKATQLGKVEVNVEQVPTSACKL
53OA        LAIDVASPGFNVAQAAMDKLTNGKATQLGKVEVNVEQVPTSACKL
            ********************************************
```

1

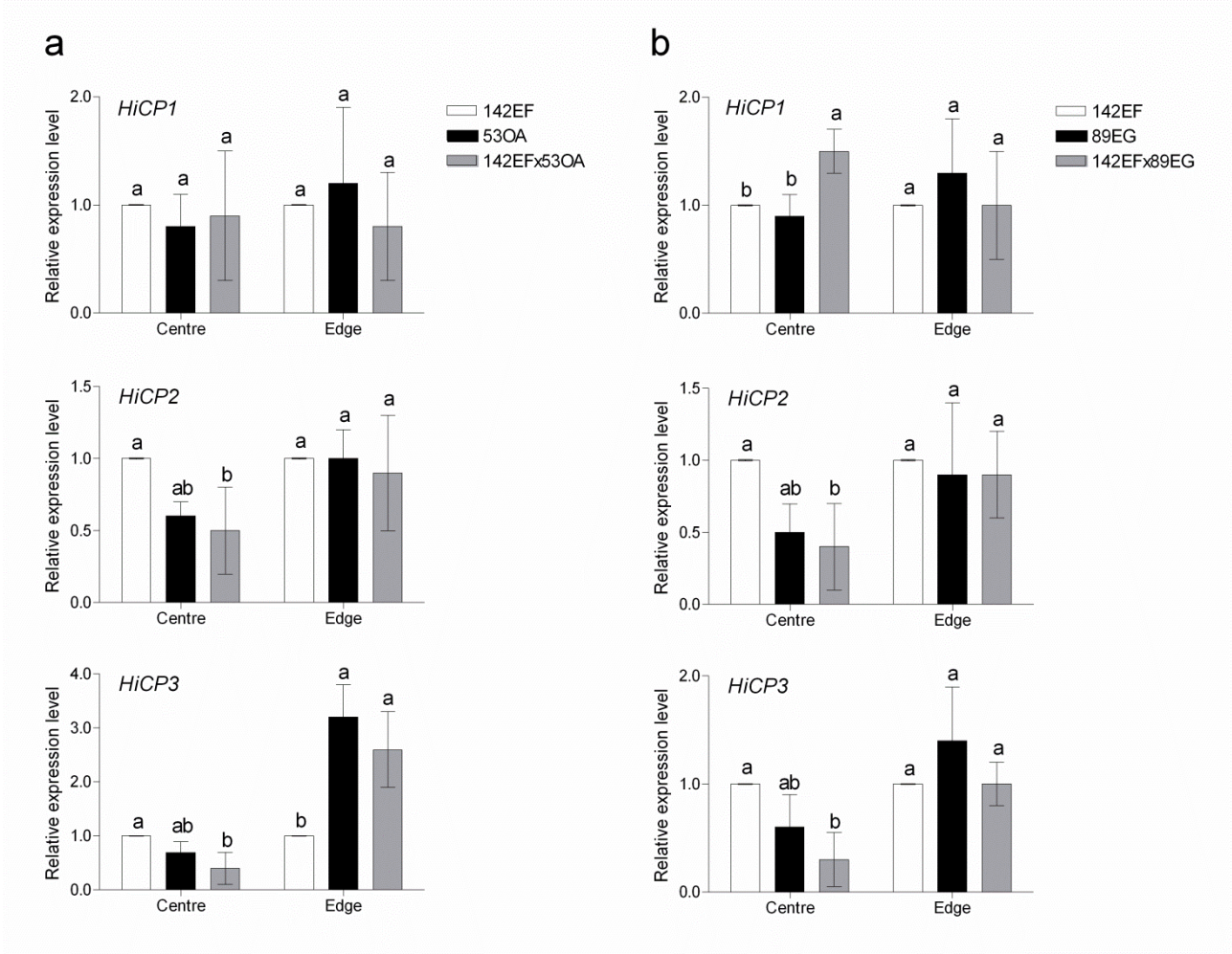
1 Fig.2


HiCP1
Edge of the colony
Centre of the colony
Relative expression level
0
1
2
3
53OA
89EG
142EF
142EFx53OA
142EFx89EG
HiCP2
Relative expression level
0
1
2
3
4
5
6
*
*
*
*
53OA
89EG
142EF
142EFx53OA
142EFx89EG
HiCP3
Relative expression level
0
1
2
3
4
5
6
7
8
9
53OA
89EG
142EF
142EFx-53OA
142EFx89EG

2

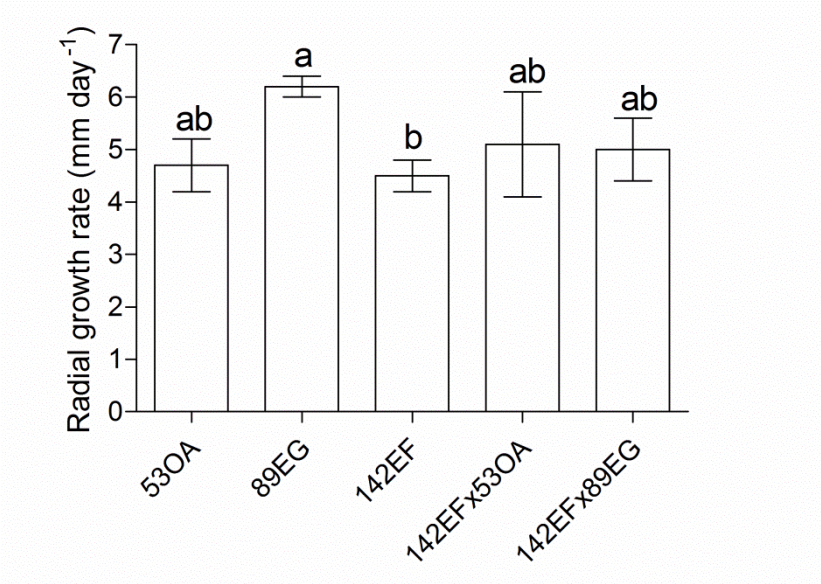
1

2 Fig.3



3

4 Fig.4


Radial growth rate (mm day-1)
0
1
2
3
4
5
6
7
ab
a
b
ab
ab
53OA
89EG
142EF
142EFx53OA
142EFx89EG

1

2