QTL FOR RESISTANCE TO *TRICHODERMA* LYTIC ENZYMES AND METABOLITES IN *AGARICUS BISPORUS.*

MARIE FOULONGNE-ORIOL, NATHALIE MINVIELLE, JEAN-MICHEL SAVOIE.
INRA, UR1264 Mycologie et Sécurité des Aliments,
F-33883 Villenave d'Ornon,
France
mfoulong@bordeaux.inra.fr

ABSTRACT

Trichoderma aggressivum leads to severe crop losses in *Agaricus bisporus* cultures. The development of strain resistant to this competitor is an alternative to the use of chemicals. One of the interacting components of the putative system of resistance is the lack of susceptibility to the growth limiting compounds produced by *Trichoderma* sp. Wide variation for this trait has been previously demonstrated, with few strains able to resist to *Trichoderma* lytic enzymes and metabolites. For exploiting such a resistance in breeding programs, the knowledge of its genetic basis is a prerequisite. Therefore, QTL analysis was used to determine the number, effects and location of genomic regions associated with tolerance to *Trichoderma* lytic enzymes and metabolites in a hybrid progeny of *A. bisporus.* An *in vitro* experiment using sequential cultures on media supplemented or not with a commercial product Lysing Enzyme® was used. The mycelium growth rate in control condition, the level of tolerance and the capacity of adaptation were the traits used for QTL detection. In total for all the traits, seven QTLs were detected distributed on four genomic regions. Two clusters of QTLs related to several traits and two other trait-specific QTLs were identified. A genomic region on LGIV was detected for each trait, with the highest LOD score value and genetic effects. Our results showed that tolerance to *Trichoderma* lytic enzymes and metabolites was tightly related to mycelium growth ability. Consequences for mushroom breeding program are discussed.

Keywords: competitor, green mould disease, adaptation, fitness, breeding

INTRODUCTION

The fungal competitor *Trichoderma aggressivum* is the cause of the green mould disease in the cultivated button mushroom *Agaricus bisporus*. It develops a dense hyphal network in mushroom compost or casing materials, followed by sporulation and an almost complete lack of mushroom production. The *T. aggressivum* species compete for space and nutrients and are adapted for growth in *Agaricus* mushroom compost by resisting the inhibiting effects of bacteria in this cultivation substrate [1]. The North American *Trichoderma aggressivum* f. *aggressivum*, produces antifungal compounds in mushroom compost that inhibit mycelial growth of the commercial mushroom *A. bisporus* which results in drastic crop losses [2]. This threat affects an ever larger part of producing areas all over the world and becomes a major disorder in commercial mushroom production [3]. Prophylactic management and disinfectant treatments may limit the epidemic but the severe level of chemical residue in food product together with the increasing risk of fungicide resistance led us to consider the cultivation of resistant *A. bisporus* varieties as a promising alternative.

Little is known about the resistance to *T. aggressivum* and its mechanism in *A. bisporus*. Although brown line formation and laccases production were described when *Lentinula edodes*

or *Pleurotus* species are confronted to *T. aggressivum*, no such defense reactions of *A. bisporus* cultivars to *T. aggressivum* attack have been observed [4, 5, 6]. Wide variations within *A. bisporus* strains in response to substrate infestation by *T. aggressivum* have been demonstrated. A few strains appeared slightly susceptible but no absolute resistance was observed [7, 8].

The evaluation of green mould resistance appeared to be quite difficult and mostly influenced by the environment [1]. Many components are involved in the interaction between *A. bisporus* and *T. aggressivum*. Some of them are due to abilities of the mushroom strains to counteract growth of moulds by producing fungistatic compounds and to resist to growth limiting compounds (enzymes, volatile compounds, small non-volatile molecules) produced by *Trichoderma* sp. In a previous study, an *in vitro* test using as media supplement a commercial product (Lysing Enzyme®) containing cell wall lytic enzymes and undefined metabolites allowed to identify strains of *A. bisporus* able to resist to this growth limitation [9]. For exploiting such abilities as a component of resistance in breeding programs, the knowledge of its genetic basis is a prerequisite.

The objective of the present work was to investigate the genetic control of the resistance to *Trichoderma* lytic enzymes and metabolites in an *A. bisporus* progeny using QTL analysis. Progresses in the understanding of this particular fungal-fungal interaction and prospects for mushroom breeding are discussed.

MATERIALS and METHODS

Offspring. The population used in our study consisted of 103 second generation hybrids obtained by crossing the homokaryotic offspring (H_i) of the hybrid JB3-83 x U1-7 (H) with the homokaryon U1-2 [10, 11, 12]. The strains JB3, U1, and the first generation hybrid H were used as controls in the experiments.

Experimental design and phenotypic evaluation. The experiment was performed according to a methodology adapted from Savoie et al. [9] and described in Fig. 1.

Cristomalt (1%) and agar (1.5%) was used as the basic media. Lysing Enzyme® (LE) from *T. harzianum* (Sigma, L1412) is a lyophilized powder obtained from cultures of *T. harzianum*, containing about 80% protein, with cellulase, chitinase and protease activities. Supplementation of Cristomalt Agar with LE was made by diluting the product in water (0.75 g in 10 mL), sterilizing by filtration (0.22 µm), and adding this solution to 1 L of autoclaved media. Un-supplemented controls contained 10 mL of sterile water.

For each strain of *A. bisporus*, a mycelium agar plug of pre-culture (5 mm-∅) was placed in the centre of Petri dishes containing malt-agar with or without LE supplementation. The inoculated media were incubated at 25 °C in the dark for 14 days and at the end of this period, the diameters of mycelial colonies were recorded (two perpendicular diameters per colony). The mean of these two values stood for mycelial growth rate parameter expressed in mm. The measures obtained from this first set of supplemented (L) or unsupplemented malt agar plates were designated as Culture 1 growth rates (C1). Culture 2 mycelial growth rates (C2) were obtained as above after taking inoculation disks from the margin of 14-day old Culture 1 plates incubated under the same conditions. Four replicate Petri dishes were inoculated for each strain in both Culture 1 and Culture 2 sets. Two additional variables were calculated: C2L/C1 and C2L/C1L [9].

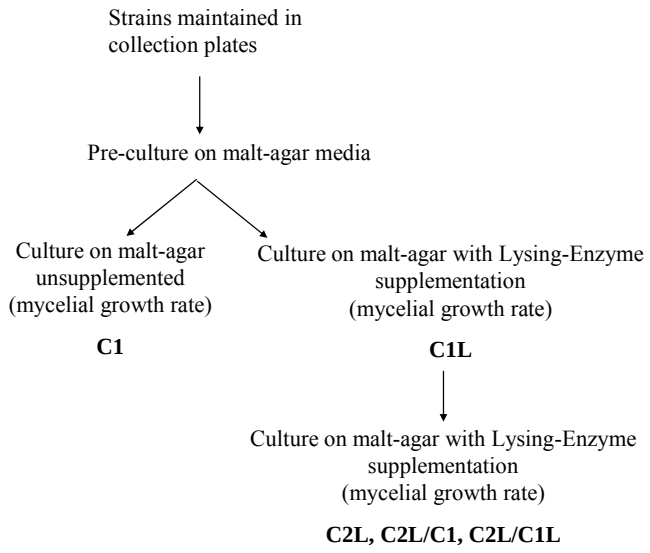

Figure 1. Methodology used to evaluate the effects of *Trichoderma* metabolites (Lysing-Enzyme) on mycelial growth rate of *Agaricus bisporus* strains, adapted from Savoie and Mata [13].

Statistical analyses and QTL detection. Means of mycelial growth were analysed by one-way ANOVAs followed by post-hoc tests for multiple comparison. Broad sense heritabilities based on genotypic mean values were estimated as $h^2 = \sigma^2_G / \sigma^2_P$ where σ^2_G represented the genetic variance, σ^2_P the phenotypic variance. Pearson correlation coefficients were calculated between each trait, based on means of each genotype. Data analyses were performed with the R-open source software.

The genetic linkage map used for QTL mapping was previously developed by Foulongne-Oriol et al. [14]. QTL detection was performed by composite interval mapping (CIM) with QTL Cartographer software [15], using B1 design. A LOD threshold of 2.5 was used to declare a QTL significant. The most likely position of each QTL was defined by the LOD score peak and a LOD-1 confidence interval. MapChart software [16] was used to produce visualisations of chromosomes carrying QTLs. The percentages of phenotypic variation explained by each QTL (R^2) and their individual additive effects (a) were given by the model. The phenotypic variation explained by all detected QTLs (R^2_T in %) was determined by multiple regression analysis, using the closest markers to the LOD score peak for each putative QTL as explanatory variables.

RESULTS and DISCUSSION

Traits evaluation. The C1 variable represented the behaviour of the mycelium in control conditions. The LE supplementation significantly affected the mycelia growth rate (Fig. 2A). Among the 103 hybrids progeny, 6% showed no significant difference between C1 and C2L, suggesting that these strains are highly tolerant to LE effects. Conversely, no lethal effect was observed. The level of tolerance of a strain could be estimated as the ratio C2L/C1 [9]. Twenty percent of the strains had similar mycelium growth rates for Culture 2 + LE and Culture 1 + LE, depicting a potential capacity of adaptation to LE effects [9]. This trait was assessed using the ratio C2L/C1L. Six percent of the strains showed higher significant mycelial growth rate on Culture 2 + LE than on Culture 1 + LE but lower than controls as illustrated in Fig. 2B.

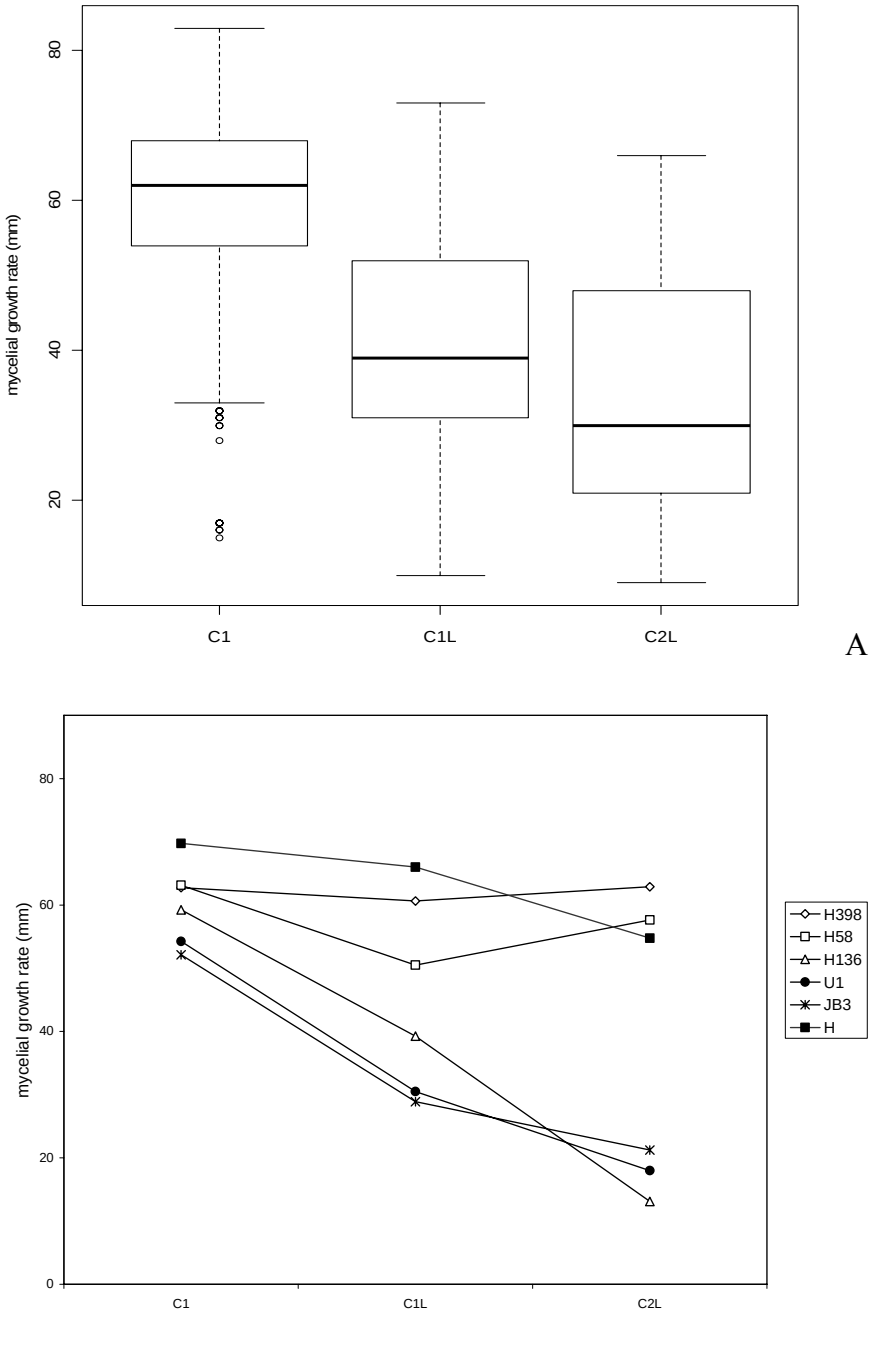

Figure 2. A- Box plot distribution of mycelial growth rate under the three tested conditions: control (C1), Culture 1 + LE (C1L), Culture 2 +LE (C2L)
B- Behaviour of some contrasted hybrids, together with the parental strains for C1, C1L and C2L

Phenotypic variation. Each trait under consideration showed a continuous distribution (Fig. 3) suggesting a quantitative inheritance. This confirmed also that the trait evaluation procedure used was reliable to assess variability in the behaviour of the hybrids progeny facing *Trichoderma* lytic enzymes and metabolites. For C1L and C2L, we observed a shift of the distribution towards the lowest values of the traits (Fig.2, Table 1), demonstrating that the LE supplementation affects mycelium growth rates. In control condition C1, 77% of the hybrids showed a mycelium growth higher than 55 mm. This percentage decreased to 38 % and 30 % for C1L and C2L respectively.

The ANOVAs revealed significant genotype effects for each trait. Post-hoc tests of ANOVAs showed that for all the traits except C2L/C1L, the two parents U1 and Jb3 were not significantly different from each other. The first generation hybrid H was found significantly better than the best parent for each trait, suggesting a strong heterosis effect. Transgressive segregants towards lowest but also in some cases highest values of trait were found among hybrids (Table 1). The broad-sense heritabilities based on genetic variance were high, ranging from 0.60 (C2L/C1) to 0.90 (C2L) indicating that the observed variation between strains is mostly under genetic control. This result was consistent with the *in vitro* experiment which allowed quite controlled environmental conditions.

The mycelial growth rates on LE supplemented media (C2L, C1L) were strongly correlated with the mycelial growth rate on control C1 (Fig. 3). The parameter of tolerance (C2L/C1) strongly correlated with the parameter of adaptation (C2L/C1L) but these two parameters were not found correlated with C1.

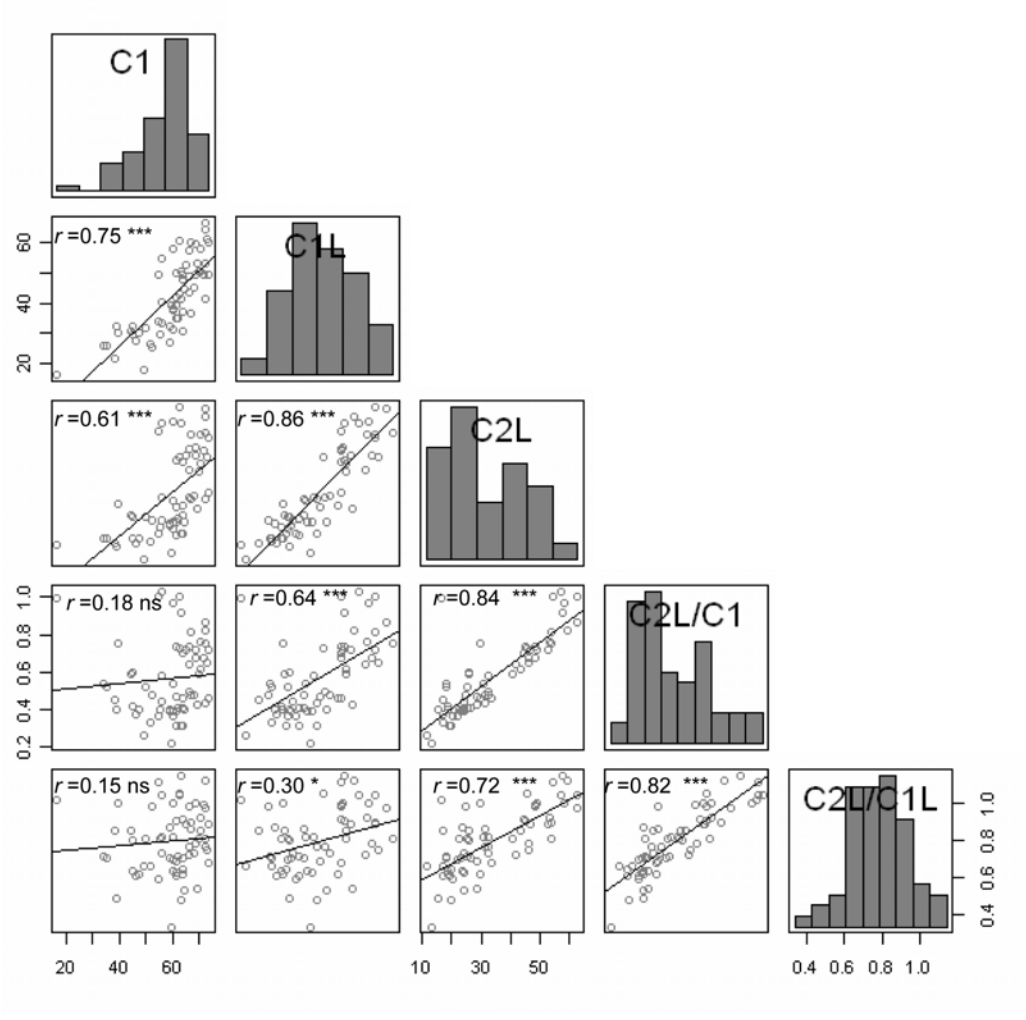

Figure 3. Frequency distribution histograms and scatter-plot matrix of *Trichoderma* metabolites resistance traits in the second generation hybrids progeny. Pearson correlation values between each pair of traits are indicated in the upper left corner of the scatter-plots (ns non significant, * significant at $p<0.05$, *** significant at $p<0.001$)

Table 1. Means, standard deviation (sd), range and heritability for *Trichoderma* metabolites resistance traits in the parental strains U1 and JB3, the H hybrid, and the second generation hybrids progeny (*n*=103).

	Parents			Offspring			
Trait	U1	JB3	H	mean	sd	range	h^2
C1	54.25 ^{B}	52.12 ^{B}	69.75 ^{A}	59.07	10.68	16.5 ^{C} - 77.16 ^{A}	0.84
C1L	30.50 ^{B}	28.87 ^{B}	66.00 ^{A}	41.27	12.43	16.12 ^{C} - 67.83 ^{A}	0.88
C2L	18.00 ^{C}	21.25 ^{C}	54.75 ^{B}	33.74	14.38	11.25 ^{D} - 64.25 ^{A}	0.90
C2L/C1	0.33 ^{C}	0.41 ^{C}	0.79 ^{B}	0.56	0.19	0.22 ^{C} - 1.03 ^{A}	0.60
C2L/C1L	0.59 ^{C}	0.74 ^{B}	0.84 ^{B}	0.79	0.17	0.33 ^{D} -1.22 ^{A}	0.63

Capital letter in exponent indicates significant means differences revealed by post-hoc ANOVA tests (p<0.05).

QTL results. A total of 3, 2 and 2 QTLs were detected using the 2.5 LOD threshold for C1, C2L/C1, C2L/C1L respectively (Table 2, Fig. 4) with individual R^2 ranging from 5.4 (QC1-I) to 15.6 % (QC1-IV). The total phenotypic variation explained by all the QTLs (R^2t) ranged from 23.4% to 26.7%. The remaining phenotypic variation which could not be explained by the detected QTL might come from other source such as QTL with too small effect to be detected in this experiment or QTL masked by differential interaction effect between segregating nuclei and the constant nucleus [17]. The number of QTL and their effects range for the mycelium growth rate in control condition (C1) were quite comparable to those found for *Pleurotus ostreatus* [17].

Table 2. Summary of QTL analyses for *Trichoderma* metabolites resistance traits

Trait	QTL name	LG	Marker[(a)]	LOD (CIM) [(b)]	additive effect	parental allele [(c)]	R^2 (%)	R^2T (%)
C1	QC1-I	I	PR007	2.9	5.33	U1-7	5.4	26.7
	QC1-IV	IV	EAAMAGt	5.3	10.3	Jb3-83	15.6	
	QC1-XIII	XIII	PR003	3.2	7.2	U1-7	8.3	
C2L/C1	QC2L/C1-IV	IV	PR041	3.7	0.2	Jb3-83	13.9	23.4
	QC2L/C1-VI	VI	ECGMCCs	2.9	0.33	Jb3-83	10	
C2L/C1L	QC2L/C1L-IV	IV	EAAMAGt	4.3	0.14	Jb3-83	15.3	23.8
	QC2L/C1L-XIII	XIII	ECGMACf	3.1	0.12	U1-7	9.4	

[(a)] nearest marker upstream to the LOD score peak
[(b)] LOD score value at the LOD score peak
[(c)] parental allele that contributes to increase the trait

Although 7 QTLs were detected for all the traits, only 4 genomic regions were highlighted (Fig. 4). Thus, the overlapping of confidence intervals revealed 2 clusters of QTLs that governed distinct traits, on LGIV and LGXIII. A genomic region of approximately 20 cM around the marker PR041 was detected for each trait (C1, C2L/C1, C2L/C1L), with the highest LOD score value. Two other genomic regions on LGI and LGVI were found each one specific to one trait, C1 and C2L/C1 respectively. These results, together with the correlation parameters suggested that either the tolerance or the adaptation were tightly linked to the growth rate ability. No significant epistatic interaction was detected.

The favourable allele that contributes to increase the trait came from either U1-7 or Jb3-83. This result was not surprising regarding the phenotypic value of each parent U1, JB3 and the H hybrid (Table 1). It suggested a complex genotypic makeup of the tolerance to *Trichoderma* lytic enzymes and metabolites. It confirmed also that potential source of resistance to green mould could be found in both wild and cultivated strains [8].

As the cap colour is a segregating trait in the studied progeny [18], our results also confirmed that the adaptation to *Trichoderma* lytic enzymes and metabolites, a trait contributing to the green mould resistance, was not related to the brown cap colour [8, 19]. Indeed, no QTL related to the traits studied here was found in the vicinity of the *PPC1* locus on LGVIII.

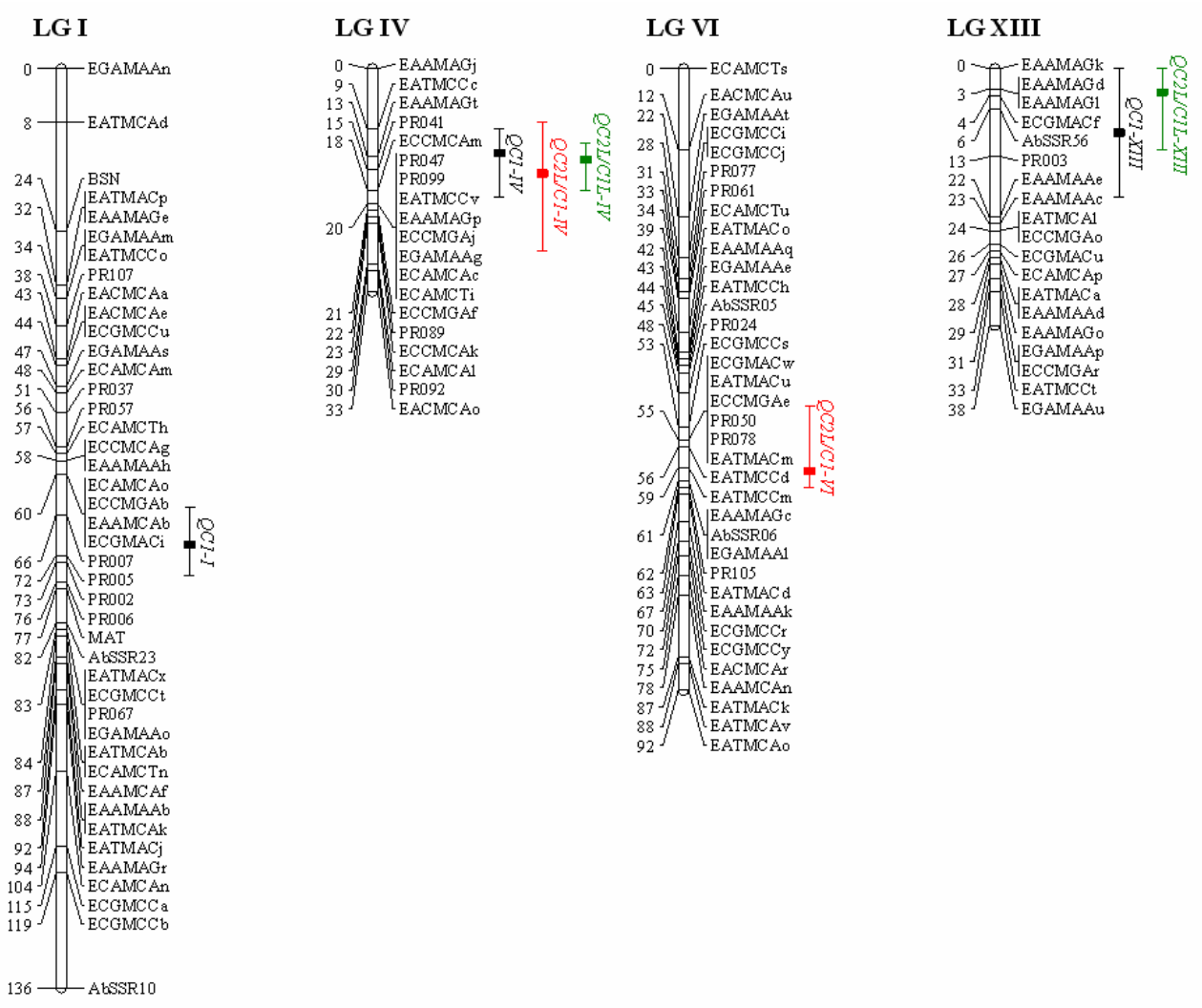

Figure 4. Map positions of the significant QTLs on the *A. bisporus* linkage map. A dash indicates LOD score peak position. The vertical bars represent the confidence intervals of the QTL (LODmax-1). QTLs for C1, C2L/C1, C2L/C1L are indicated in black, red and green respectively.

CONCLUSIONS

This was the first study dealing with QTLs related to resistance to the green mould disease in basidiomycetes. The procedure used here can be applied to other *Trichoderma*-fungus interaction to identify genomic regions involved in the resistance to this competitor causing significant damages during cultivation of many mushroom species.

We have demonstrated that the ability to resist to *T. harzianum* lytic enzymes and metabolites in *A. bisporus* was quantitatively inherited and under oligogenic control. Besides, we have identified one robust QTL on LGIV that lets presume a key role played by this particular part of the genome. The recent release of the *A. bisporus* genome sequence will help to go further in the understanding of the growth rate control.

Our results suggested that the genetic factors involved in the ability to resist or adapt to *Trichoderma* lytic enzymes and metabolites are linked to the fitness of the *A. bisporus* strains. Both traits might be involved in resistance to the green mould disease. The incidence of severity of the disease might be reduced if *A. bisporus* successfully colonizes the compost before *T. aggressivum* develops [4, 20]. During infection, at the contact point between the two fungi, the strains of *A. bisporus* having the ability to resist or to adapt to the lytic enzymes and metabolites with antibiotic effects produced by *T. aggressivum* are better armed to be less affected than others.

Further work is needed to correlate closely the present laboratory test to the resistance to *T. aggressivum* in compost based cultivation systems, but our results offer opportunities to breed and select for *A. bisporus* strains with increased potentials to outcompete the green mould.

REFERENCES

[1] Savoie J.M. *et al.* (2001). Factors influencing the competitive saprophytic ability of *Trichoderma harzianum* Th2 in mushroom (*Agaricus bisporus*) compost. *Mycol Res*. 105: 1348-1356.

[2] Krupke O.A. *et al*. (2003). The North American mushroom competitor, *Trichoderma aggressivum* f. *aggressivum*, produces antifungal compounds in mushroom compost that inhibit mycelial growth of the commercial mushroom *Agaricus bisporus*. *Mycol Res*. 107: 1467-75.

[3] Hatvani L. *et al.* (2007). Green mold diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* Species. *Phytopathology*. 97: 532-7.

[4] Mamoun M. *et al.* (2000). Interactions between the pathogen *Trichoderma harzianum* Th2 and *Agaricus bisporus* in mushroom compost. *Mycologia*. 92: 233-240.

[5] Mumpuni A. *et al.* (1998). Effect of metabolites produced by *Trichoderma harzianum* biotypes and *Agaricus bisporus* on their respective growth radii in culture. *Appl Environ Microbiol*. 64: 5053-6.

[6] Savoie J.M. *et al.* (2001). Variability in brown line formation and extracellular laccase production during interaction between white-rot basidiomycetes and *Trichoderma harzianum* biotype Th2. *Mycologia*. 93: 243-248.

[7] Kerrigan R.W. (2004). Trait diversity in wild Agaricus bisporus. *Mushroom Sci*. 16: 31-38.

[8] Mamoun M. *et al.* (2000). Green mould disease in France: *Trichoderma harzianum* Th2 and other species causing damages on mushroom farms. *Mushroom Sci*. 15: 625-632.

[9] Savoie J.M. *et al.* (2008). Susceptibility of European populations of *Agaricus bisporus* to *Trichoderma* metabolites. in *Sixth International Conference on Mushroom Biology and Mushroom Products*. Bonn, Germany.

[10] Callac P. *et al.* (1998). *Bsn-t* alleles from French field strains of *Agaricus bisporus*. *Appl Environ Microbiol*. 64: 2105-10.
[11] Imbernon M. *et al.* (1996). *BSN*, the primary determinant of basidial spore number and reproductive mode in *Agaricus bisporus*, maps to chromosome I. *Mycologia*. 88: 749-761.
[12] Kerrigan R.W. *et al.* (1994). The heterothallic life-cycle of *Agaricus bisporus* var *burnettii* and the inheritance of its tetrasporic trait. *Exp Mycol*. 18: 193-210.
[13] Savoie J.M., G. Mata (2003). *Trichoderma harzianum* metabolites pre-adapt mushrooms to *Trichoderma aggressivum* antagonism. *Mycologia*. 95: 191-9.
[14] Foulongne-Oriol M. *et al.* (2010). An expanded genetic linkage map of an intervarietal *Agaricus bisporus* var. *bisporus* x *A. bisporus* var. *burnettii* hybrid based on AFLP, SSR and CAPS markers sheds light on the recombination behaviour of the species. *Fungal Genet Biol*. 47: 226-236.
[15] Basten C.J. *et al.* (2004). QTL Cartographer, version 1.17. Department of Statistics, North Carolina State University, Raleigh, NC.
[16] Voorrips R.E. (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. *J Hered*. 93: 77-78.
[17] Larraya L.M. *et al.* (2002). Quantitative trait loci controlling vegetative growth rate in the edible basidiomycete *Pleurotus ostreatus*. *Appl Environ Microbiol*. 68: 1109-14.
[18] Callac P. et al. (1998). Evidence for *PPC1*, a determinant of the pilei-pellis color of *Agaricus bisporus* fruitbodies. *Fungal Genet Biol*. 23: 181-188.
[19] Guthrie J.L., A.J. Castle (2006). Chitinase production during interaction of *Trichoderma aggressivum* and *Agaricus bisporus*. *Can J Microbiol*. 52: 961-7.
[20] Beyer D.M. *et al.* (2000). Evaluation of epidemiological factors and mushroom substrate characteristics influencing the occurrence and development of *Trichoderma* green mold. *Mushroom Sci*. 15: 633-640.