**Detection of a hypersensitive reaction in the chestnut hybrid 'Bouche de Bétizac' infested by Dryocosmus kuriphilus Yasumatsu**

# Detection of a hypersensitive reaction in the chestnut hybrid 'Bouche de Bétizac' infested by *Dryocosmus kuriphilus* Yasumatsu

Francesca Dini[§ 1], Chiara Sartor[§ 1], Roberto Botta [1]

[1] *University of Turin, Department of Arboriculture and Pomology, Via Leonardo da Vinci 44, Grugliasco 10095 (TO), Italy*

§: both authors contributed equally to this work.

Tel. 011-6708740

Fax. 011-6708658

E-mail: roberto.botta@unito.it

The purpose of the study was the identification of the mechanisms of resistance to *Dryocosmus kuriphilus* Yasumatsu in the hybrid resistant cultivar 'Bouche de Bétizac' (*C. sativa* x *C. crenata*). Larvae and eggs of the insect are found in the buds of this cultivar at the end of winter, but there is no gall development after budburst. The hypothesis of the presence of a hypersensitive reaction (HR) in the buds was tested using diaminobenzidine (DAB) to detect $H_2O_2$ and by Real Time PCR (RT-PCR) to evaluate the expression of a germin-like protein gene. HR in plants is elicited by the production of reactive oxygen compounds, such as $H_2O_2$, and results in the programmed cell death. The DAB test was applied to buds of 'Bouche de Bétizac' and of the susceptible cultivar 'Madonna' (*C. sativa*) at different stages of budburst. The DAB staining produced brown areas in the swelling buds of 'Bouche de Bétizac', indicating the presence of $H_2O_2$. On the contrary, all uninfested buds, as well as the infested buds of 'Madonna', appeared whitish. Papers report of germin and germin like proteins (GLP) with oxalate oxidase activity are discrete markers of stress-responsive gene products. A strong expression of the chestnut GLP gene was detected by RT-PCR at bud swelling in infested 'Bouche de Bétizac' and not in 'Madonna'. The results support the hypothesis of the occurrence of a HR in 'Bouche de Bétizac' as response to the cynipid infestation, resulting in cell and larvae death.

***KEYWORDS**: Castanea, cynipid, diaminobenzidine, DAB, germin-like protein, GLP*

Abbreviation: DAB 3,3'-diaminobenzidine; HR hypersensitive reaction; GLP germin like-protein; $H_2O_2$ hydrogen peroxide.

# 1 INTRODUCTION

The chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), is currently one of the most damaging pests of *Castanea* spp. [1]. The species is univoltine and thelytokous. Adults lay eggs in chestnut buds in early summer, and larvae remain latent until buds expand the following spring. Larvae induce the formation of leaf and twig galls with a reduction of fruit yield by 50%–75% [2,3]. The chemical control has no effect because it is limited by the cryptic nature of the insect, which lives in dormant buds for the majority of its life cycle, and by the difficulty of precisely detecting adult emergence. At moment, there are only two practical solutions to this problem: bio-control, by introducing the parasitoid *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) from China's mainland, and the genetic selection of resistant cultivar.

In Japan the work of selection and breeding of genotypes resistant to gall wasp within the germplasm of *Castanea crenata* Siebold & Zucc. began in 1947 and yielded four resistant cultivars [4]. Two of these cultivars, 'Tzukuba' and 'Tanzawa', together with the resistant cultivar 'Ginyose' still remain the most widely grown in Japan [5]. Similar studies are in progress in USA, where scientists are using alternative sources of genetic material such as *Castanea mollissima* Blume, *Castanea pumila* (L.) Mill. and *Castanopsis henryi* Skan to introduce new resistance factors into the American chestnut *Castanea dentata* Marshall [6].

In 2002 the gall wasp was found in Piedmont, North-west Italy [7]; this was the first report about this pest in Europe and since then it has quickly spread throughout Italy and is currently present also in France, Slovenia and Croatia.

In 2003 a project aimed at establishing biological control using *T. sinensis* and selecting resistant individuals was started in Piedmont. During the first years of research it was found that the Euro-Japanese hybrid 'Bouche de Bétizac' (*C. sativa* Miller X *C. crenata*) is completely resistant to the gall wasp [8]. Japanese authors reported that in the cultivars of *C. crenata* there is no preference in oviposition selection, between resistant and susceptible varieties; yet, at budburst the pest death occurs at level of the larval chamber in the resistant genotypes [9]. The mechanism underlying the resistance of the hybrid 'Bouche de Bétizac' was unknown but it was observed that its dormant buds may contain larvae or eggs, in spite of the absence of gall development after budburst.

The large amount of studies, already conducted to explain how the plant defenses act against the pathogens, reveal the presence of diverse strategies. Plants have evolved mechanisms to perceive pathogen attacks and to translate that perception into an adaptive response. So each individual plant cell must possess a preformed and/or inducible defense capability. One type of plant resistance is called gene-for-gene relationship [10]. This requires the involvement of specific molecules, called elicitors, responsible for the activation of a genotype-specific way typology of defense. However non-host disease resistance, NHL, is the most common form of resistance exhibited by plants against pathogens, in both monocot and dicot species. It is displayed at the species level (i.e. all genotypes of a plant are resistant to infection by all genotypes within a pathogen species). NHL is classified into two types [11]: type I, which is not producing any visible symptom, and type II, which is always associated with brown spots in the tissue interacting with the pathogen, because of a rapid and localized hypersensitive response (HR). HR is an induced resistance that can be detected in the area immediately adjacent to the site of attack and shows up as a circular, brown, necrotic spot [12]. This response is developed in 4 phases: recognition of pathogen attack, development of oxidative burst, activation of defense gene, cell death manifested with the appearance of necrotic tissue.

In the case of plant response to herbivorous insects, two different typologies of resistance have been reported: constitutive and induced resistance [13]. Both types are characterized by the production of compounds, such as jasmonate, oligogalacturonic acid or hydrogen peroxide. Yet, constitutive resistance is pathogen-specific while induced

resistance in not . The synthesis of hydrogen peroxide is well documented in the interaction between corn earworm (*Helicoverpa zea* Boddie) and soya bean, where it acts as component of induced resistance [14].

Further evidence shows that high levels of hydrogen peroxide are implicated in the induction of cell death in the hypersensitive response to pathogens [15]. The main components of HR passing signals are the reactive oxygen species (ROS), such as superoxide anion ($O_2$–) and hydrogen peroxide ($H_2O_2$), whose main function is to strongly amplify the signal. ROS at interaction sites may take different roles depending on the type of stimulus. In particular, they act as factors playing a key role in the defense response by: direct killing of the pathogen [16]; modifying the cell wall, to impede pathogen penetration through peroxidase-catalyzed crosslinking of polymers [17]; acting with transmission functions to activate defense responsive genes [18]; inducing hypersensitive cell death [19,20]; inhibiting the metabolism, and even disrupting the hormonal control of developmental processes [13].

The presence of HR in plants as reaction against herbivorous insects has been documented in rice against the Asian rice gall midge *Orseolia oryzae* [21]; in a resistant cultivar HR synthoms include premature tillering, browning of central leaf, and tissue necrosis at the apical meristem.

Over the last few years, several proteins potentially involved in the resistance reaction to pathogens have been identified in plants. Among them are germin and germin-like proteins (GLPs) that belong to a class of developmentally regulated glycoproteins. This group of proteins exhibit a broad range of diversity in their occurrence and activity in organisms ranging from myxomycetes, bryophytes, pteridophytes, gymnosperms and angiosperms. Germins and GLPs are thought to play a significant role during zygotic and somatic embryogenesis (wheat and *Pinus spp*, respectively) [22], salt stress (barley and *Mesembryanthemum crystallinum*), pathogen elicitation (wheat and barley), heavy metal stress [23]. Proteins belonging to this family are involved in many regulatory processes and can be divided into three groups based on their function: enzymatic (oxalate oxidase or superoxide dismutase), structural or of receptions [24]. It is thus increasingly obvious that germins and GLPs participate in many processes that are important for plant development and defence.

Previous studies proved that GLPs are discrete markers of stress-responsive gene products [25]. The role of GLP proteins with oxalate oxidase (OXO) activity was studied in wheat, barley and sunflower; these studies have clearly demonstrated how these proteins play a fondamental role in the realization of a defensive response, which occurs through the hypersensitive response [26]. The best characterized GLP, is the wheat OXO, also known as germin, that belongs to the cupin family [27]. These proteins are one of the enzymes that can produce $H_2O_2$ in plants by converting oxalic acid (OA) and $O_2$ to $CO_2$ and hydrogen peroxide ($H_2O_2$).

3.3-diaminobenzidine (DAB) is used in histochemistry to detect in vivo and in situ $H_2O_2$ due to the reaction of polymerisation of DAB in presence of hydrogen peroxide. The polymerised form is insoluble and is characterized by a brown coloration. DAB staining was used to detect the accumulation of $H_2O_2$ in response to the interaction barley–powdery mildew (*Blumeria graminis f.sp. hordei* Bgh; [28] and tomato–nematodes (*Meloidogyne incognita* Chitwood) [29].

The present work was aimed at studying the mechanism of response to the chestnut gall wasp in the resistant hybrid ‘Bouche de Bétizac‘. Even though DAB staining has been performed so far only on leaves or leaf sections, in the current research the technique was used to detect the presence of $H_2O_2$ in chestnut buds of ‘Bouche de Bétizac‘ and to prove the occurrence of the hypersensitive response in this cultivar and not in the susceptible control variety ‘Madonna’ (*C. sativa*). The results obtained by DAB staining were tested at molecular level analyzing the gene expression of a putative germin-like-protein gene of *C. crenata* by Real-Time PCR.

# 2 MATERIALS AND METHODS

**2.1 Bud collection:** In 2011, buds from cultivar 'Madonna' (C. sativa) and the hybrid 'Bouche de Bétizac' (*C. sativa* 'Bouche Rouge' X *C. crenata* CA04) were collected from single plants at different times of budburst from April 21st to May 12th. The harvest was carried out once a week in order to gather material representative of the different stages of bud sprouting and be able to observe the onset of the defensive response. The Madonna samples were collected on field in Baldissero d'Alba, near Cuneo, while the 'Bouche de Bétizac' samples derived from Chiusa Pesio; both of these areas are located in the province of Cuneo. The areas of samples were highly infested by the cynipid and plant identity was checked by DNA analysis.

The following codes M1, M2, M3, M4 and B1, B2, B3, B4 were assigned to samples from 'Madonna' and 'Bouche de Bétizac', respectively; numbers corresponded to 4 stages of development: 1=closed bud; 2= bud that initiates to swell; 3= end of bud swelling: scales separated; 4= brown scales fallen, bud enclosed by green scales. The letters "I" and "N" were used to mark infested or not infested samples, respectively. The buds for the RNA extraction were frozen in liquid nitrogen, immediately after being detached from the branch, and kept at -80°C until use. The material used for DAB staining was stored at 4°C during transportation and treated in the same day of sampling.

**2.2 Cytochemical localization of $H_2O_2$:** DAB protocol was used to detect the presence of hydrogen peroxide following the larva development in the resistant genotype. The method was set up specifically for the staining of the compact tissues of the chestnut buds. Buds were placed in a solution of DAB (1 mg/ml), 0.05 M Tris-HCl and 0.15 M NaCl (pH 3.8) [30] and vacuum-infiltrated for 20 minutes [31], then left in the same solution for 8-10h in darkness. Samples were boiled in 99% ethanol for about 30 minutes to remove all traces of DAB and chlorophyll. Buds were preserved in ethanol 70% and observed under stereomicroscope after the progressive removal of scales. The presence of hydrogen peroxide was revealed by the formation of a brown insoluble precipitate in the tissue.

**2.3 Nucleic Acids extraction:** The expression of a germin like-protein of *C. crenata* was studied in the samples to validate the result obtained by the DAB protocol. Nucleic acids extraction was performed from single buds at stages 2 and 4 of budburst . Buds were disrupted in liquid nitrogen using a baked mortar and pestle treated with DEPC water. Nucleic acids were extracted using a buffer containing: 2% CTAB, 2% polyvinylpyrrolidone (PVP) K-30 (soluble), 100 mM Tris HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine, 2% β-mercaptoethanol. After two buffered chloroform extractions, the upper phase containing the nucleic acids was divided in two parts for RNA and DNA extraction. DNA was precipitated with 0.7 volumes of isopropanol and washed in 70% ethanol; it was dried and resuspended in 50 µl of sterile water.

Total RNA was precipitated overnight with 8M LiCl at 4°C. The next day RNA was added of a SSTE buffer (5.0 M NaCl, 0.5% SDS, 10 mM Tris HCl pH 8.0, 1 mM EDTA), and treated at 65 °C for 10min. Following two chloroform purifications, RNA was precipitated and then washed in Ethanol 100% and 70%. Total RNA was purified with RNeasy Mini Kit (Quiagen). RNA yield and quality were evaluated using spectrophotometric determinations. Overall 6 replicates were extracted for each stage of bud development for both 'Bouche de Bétizac' and 'Madonna'.

**2.4 Cynipid Diagnostic PCR:** A diagnostic PCR was performed to recognize between healthy and infested buds. This assay identifies buds containing the insect through the selective amplification of the wasp 28S Ribosomal

DNA [32]. DNA extracted from buds was amplified by a two step PCR. The PCR mix (20 µl) consisted of 5 µl of the extracted DNA (about 250 ng), 0.5 U of BIOTAQ polymerase (Bioline, London UK), 2 µl of buffer 10X, 0.9 µl of $MgCl_2$ 50 mM, 1 µl of forward and reverse primer mix (20 pM/µl), 0.2 µl dNTP mix (20 mM each), and 10.8 µl sterile water. The first PCR included 3 minutes of denaturation at 95°C, followed by 37 cycles of 3sec at 95°C, 4sec at 55°C (for 37 cycles), 1 min at 72°C; a final extension step was performed at 72°C for 10 min. The second PCR used 2 µl of the amplification product as template and was carried out for 28 cycles at the same conditions as the first PCR. Amplification products were run on 1% agarose gel and visualized with a UV transilluminator, after ethidium bromide staining.

**2.5 Real time PCR :** Once samples were recognized as infested (I) or uninfested (U), cDNA synthesis was performed in duplicate using 3µg of total RNA from the 2 cultivars (B, M), separately for infested and not infested samples, at stages 2 and 4 of bud shooting. RNA was treated with Dnase I, RNase-free (Fermentas), and reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Genes for GLPs proteins, showing oxalate oxidase activity, reported as being involved in HR response were selected by literature and NCBI database search. GLP sequences were found in *Arabidopsis thaliana, Ordeum vulgare, Sinapis alba, Pharbitis nil, Lupinus albus, Oryza sativa, Triticum aestivum, Pinus caribea* and *Quercus robur* .

The GLP cDNA sequences found in the NCBI database were compared with the chestnut transcriptome by the BLAST service of the *Fagaceae* Genomic Web site**,** where all the outputs of the chestnut new generation sequencing are collected.

Specific primers for the gene encoding for a germin like-protein (GLP1-F:5'-CGAGGCTCCTTGTGACACTT-3'and GLP1-R:5'-AGGGAGGTCCAATCACATCA-3') were designed on the sequence "CC454_contig29828" found in the *Fagaceae* Genomic Web (*www.fagaceae.org* ) site and obtained by Roche 454 pyrosequencing of the *Castanea crenata* transcriptome. .

VvUbiquitin1 was chosen as housekeeping gene (primers: F-Ubi: 5'-TCTGAGGCTTCGTGGTGGTA-3' and R-Ubi: 5'-AGGCGTGCATAACATTTGCG-3') [33]. The relative expression of the germin like-protein gene was computed using the Relative Standard Curve Method to be sure to detect weak expression levels. The geometric mean of the expression ratios of the housekeeping gene was used as the normalization factor in all samples. Quantitative PCR was performed with StepOne Plus Real Time PCR system (Applied Biosystems) in a reaction volume of 20 µl containing 10µl of PowerSYBR Green master mix (Applied Biosystems), 0.25µM of each primer, and 2µl of cDNA diluted 1:10. Cycling conditions for all primer pairs consisted of an initial denaturation at 95°C for 10min, followed by 40 cycles at 95°C for 15s, and 60°C for 1 min. PCR was performed in triplicate, and specific annealing of the primers was checked on dissociation kinetics performed at the end of each PCR run. Gene expression was expressed as the mean and standard error calculated over all biological and technical replicates.

# 3 RESULTS

**3.1 Identification of infested buds by diagnostic PCR:** The diagnostic PCR allowed to identify the bud samples containing cynipid larvae producing a specific amplicon of 320 bp [32]. The sensitivity of the test is very high being able to detect one larva in 2 g of tissue, i.e. in 8-10 buds. The analysis was replicated 2 times and positive and negative samples were separated and further used for the Real Time analyses.

**3.2 Detection of hydrogen peroxide presence in buds using the Diaminobenzidine staining:** 3,3'-diaminobenzidine (DAB) staining was performed on 'Bouche de Bétizac' and 'Madonna' buds, at different stages of development, to determine the presence of $H_2O_2$. The appearence of brown staining reveals in situ DAB polymerization driven by $H_2O_2$. Our results showed the presence of a reaction of polymerization in 'Bouche de Bétizac' buds and not in 'Madonna' ones.

'Madonna' buds appeared internally whitish (Fig.1A), both in not infested (N) and infested (I) buds (Figs. 1B, 1C). At the first stages M1-I and M2-I, eggs of the wasp were found in the inner part of the bud (Fig.1D), while at stage M3-I the larval chambers were observed (Fig.1E). The larval chamber, which is structurally similar in all cynipid galls [34], is lined with nutritive plant tissue of which the larva feeds, and is surrounded by a thin wall of sclerenchyma [35]. The cynipid larva completes its entire development within this chamber. Gall primordia were observed at the fourth stage, M4-I, in the inner layers of the bud (Fig. 1F), with the wasp at the larval stage (Fig. 1G).

The uninfested buds of 'Bouche de Bétizac' treated with the DAB solution were internally whitish due to the lack of $H_2O_2$ reaction (Figs. 2A, 2B). Inside the ovideposited buds, wasp eggs (B1-I, B2-I) and larvae (B3-I) were found at the early stages (Fig. 2C, 2D) while at stage B4-I both gall primordia and large dark brown-stained areas, due to DAB polymerization, were observed (Figs.2E, 2F). Such areas appeared to be undergoing tissue degeneration, probably due the starting of cell death (Fig.2G) revealed by the easy disruption of the tissue by compression during the examination.

**3.3 Testing of GLP expression levels in the susceptible and the resistance cultivar:** A molecular analysis was carried out to support the results obtained with DAB staining. Following the information got from the DAB staining, our goal was to reveal by RealTime PCR different expression levels of a GLP gene in infested (I) and not infested (N) buds at stages 2 and 4 of budburst in 'Bouche de Bétizac' (B) and 'Madonna' (M).

The results of Real Time PCR showed a level of expression of the GLP gene significantly higher in 'Bouche de Bétizac' infested samples at stage B2-I, in comparison with the other three cases (Fig 3). In particular, infested stage B2-I revealed an expression 25 times greater than in M2-I of 'Madonna' and 5 times higher than in B4-I of the same 'Bouche de Bètizac'. Although the expression levels in 'Madonna' were much lower than in 'Bouche de Bétizac' they showed a similar pattern across the samples. In infested 'Madonna' at stage M2-I, GLP gene expression was twice as much the same stage in not infested buds. In both cultivars the lowest value of expression was observed at the stage 4-N. The hypothesis that the defense response to the cynipid in 'Bouche de Bétizac' is of hypersensitive type is reinforced by these results.

# 4 DISCUSSION

The recent introduction of the gall wasp (*Dryocosmus kuriphilus* Yasumatsu) in Italy is causing severe damages and yield losses and rapid solutions are urgently required. In Japan, before the spread of the parasitoid *Torymus sinensis* Kamijo, resistant genotypes were found in *Castanea crenata* and used to breed new cultivars. Since a biotype of the insect was eventually able to overcome the resistance, in the following years the strategy was joined with the biological control, using the natural enemy *Torymus sinensis*,. The release of the parasitoid started in 1982 and the effective containment of the chestnut gall wasp was reached after a variable period ranging from 6 to 20 years, depending on the site of release [2].

Resistance to chestnut gall wasp is reported to be present in other *Castanea* species (*Castanea mollissima*, *Castanea pumila*) but not yet in *C. sativa*. The studies on the bases of resistance agree on the hypothesis that more mechanisms may be responsible of the resistance, or tolerance, in the different genotypes of chestnut [4,6].

In Italy, the work of selection for resistance has been carried out prevalently on the local *Castanea sativa* germplasm [36] but the first fully resistant cultivar found was the Euro-japanese hybrid 'Bouche de Bétizac' [8].

The aim of this work was to understand the mechanism of response to gall wasp in the resistant 'Bouche de Bétizac' and in the cultivar 'Madonna' (*C. sativa*), showing medium-high susceptibility [8], at onset of gall formation when the resistant reaction occurs in 'Bouche de Bétizac'.

The occurrence of the hypersensitivity reaction against galling herbivores insects was described by Fernandes [37] who reported that the hypersensitive response can be considered the most common plant resistance mechanism against phytophagous. He wrote that the under-detection of this typology of resistance in plants is due to the apparent lack of symptoms, since these have been mistaken for spots caused by diseases. Fernandes [38] observed that HR in a genotype resistant against a galling insect appears as a round spot around the gall initiation site on the leaf; he suggested that the necrosis is due to an upset in the balance between oxidative and reductive process, leading to the oxidation of phenolic compounds and the breakdown of cells and cell compartments . In chestnut buds affected by the gall wasp, Shimura [2] observed similar symptoms, i.e. the early necrosis of the tissue surrounding the larval chamber resulting in the death of the larvae, in the cultivar 'Ginyose' (*C. crenata*), resistant to *D. kuriphilus*. The histochemical observations conducted in this work clearly revealed the presence of $H_2O_2$ in the infested buds of 'Bouche de Bétizac' at a specific stage of budburst (B4-I). In addition, the development of $H_2O_2$ in the tissue of the larval chamber was specific of the responsive reaction: 'Madonna' buds containing larval cells in growth did not show DAB staining, thus larva feeding does not cause $H_2O_2$ release. The brown spots developed by the DAB-$H_2O_2$ reaction in the inner part of the bud support the hypothesis of the presence of a hypersensitive reactions involved in the mechanisms of 'Bouche de Betizac' resistance.

This is the first work that detects the presence of a hypersensitive reaction at bud level; since until now it has been observed only in leaves. At the same time, it is the first paper using the DAB reaction for detecting $H_2O_2$ in buds, as it was previously used only on leaves or young plants.

The hypothesis concerning the presence of a HR in 'Bouche de Bétizac' was reinforced by the results of the molecular test performed by RealTime-PCR at different stages of budburst in 'Bouche de Betizac' and 'Madonna'. The RT-PCR showed different levels of expression of the putative GLP gene. As previously described GLP proteins, with oxalate oxidase activities, are involved in the production of hydrogen peroxide, a compound found at the initial phase of the hypersensitive response. The expression of the GLP gene of chestnut was very high in infested 'Bouche de Bétizac' tissues at stage B2-I of budburst, a level that suggests the onset of a massive defensive response just when buds initiate

to swell. In B4-I the level of expression was lower than in B2-I but remained still higher than in the healthy buds of 'Bouche de Bétizac' (B2-N, B4-N) and in all samples from 'Madonna'.

These observations allow to recognize the particular time when the mechanism of resistance starts and to identify one of the genes involved in the process. Molecular and histological results provide a good view of the mechanism at work: the HR in 'Bouche de Bétizac' started in B2-I with the transcription of the enzyme responsible for the synthesis of hydrogen peroxide from oxalate. The product of the enzymatic activity was found 2 weeks later by DAB staining, when the molecular evidence showed that the activity of the GLP gene had decreased but was still higher than in unifested buds. At the same time these results show the stage of development of the bud when the hypersensitive response activated by 'Bouche de Bétizac' kills the insect. Stage 4 shows an intact gall primordia (on the right side) containing living larvae besides a larval cell already undergoing HR (left side). This indicates that B4 is the turning stage when the wasp larvae start to die in 'Bouche de Bétizac' buds. As reported by Quacchia [40], the first-instar larvae overwinter and grow slowly until the following spring when their growth rate increases leading to the induction of gall development. Larvae reach the second instar just

The date of sampling of stage 4 buds was May 12$^{th}$, 2011, when larvae were still at first-instar.

Even if this work does not reveal in detail how the resistance process against the chestnut gall wasp is structured, the hypothesis of the presence of HR in 'Bouche de Bétizac' appears to be in accordance with findings of other papers concerning the study both of gall insect resistance [37,38] and of other types of pathogens, such as the chestnut blight (*Cryphonectria parasitica* Murr.), affecting chestnut [39]. Barakat [39] compared the transcriptomes of the American chestnut (*C. dentata*), blight sensitive, and the Chinese chestnut (*C. mollissima*) in response to infection by *Cryphonectria parasitica* and identified proteins, expressed in the resistant species, involved in HR cell death. The current results and the observations made on 'Ginyose' by Shimura [2], suggest that HR developed by de 'Bouche de Bétizac is due to the genetic component arising from the *C. crenata* parent, the INRA selection CA04; in fact the *C. sativa* parent 'Bouche Rouge' was found infested in orchards (Hennion, personal communication).

# 5 CONCLUSIONS

This work demonstrate the presence of a hypersensitive response to *D. kuriphilus* in the Euro-Japanese hybrid 'Bouche de Bétizac'. The genetic bases of the mechanism require further investigations and are very likely related to the *C. crenata* parent. In Japan different sources of resistance were found in *C. crenata* and a biotype of the insect was able to overcome some of them. In Italy, ten years after the first report on the presence of the insect, 'Bouche de Bétizac' is still fully resistant. Yet, the work of selection aimed at finding more sources of resistance in *C. sativa* is continuing and has recently revealed the existance of resistant genotypes both within cultivars and wild germplasm. The present results will contribute to study these individuals and to understand the genetic origin of the response, with the final goal of breeding new cultivars belonging to *Castanea sativa*.

## Aknowledgements

The research was funded by Regione Piemonte Administration.

# REFERENCE


[1] Y. Abe ,G. Melika, G.N. Stone. The diversity and phylogeography of cynipid gallwasps (*Hymenoptera, Cynipidae*) of the Eastern Palearctic and their associated communities. Oriental insect 41 (2007) 169-212.

[2] J.A. Payne ,R.A. Jaynes and S.J.Kays. Chinese chestnut production in the United States: practice, problems and possible solutions. Econ. Bot. 37(1983)187–200.

[3] K. Kato and N. Hijii. Effects of gall formation by *Dryocosmus kuriphilus Yasumatsu* (Hym., Cynipidae) on the growth of chestnut trees. J. Appl. Entomol. 121(1997)9–15.

[4] I Shimura. Studies on the breeding of chestnut, *Castanea* spp. II. Parasitic variation in the chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu. Bulletin of the Horticultural Research. Station A11(1972) 1-13 (in Japanese with English summary).

[5] G. Bounous, G. Beccaro. Chestnut Culture: Directions for Establishing New Orchards. Nucis – Information Bulletin of The Research Network on Nuts (FAOCIHEAM)11 (2002) 30–34.

[6] S. Anagnostakis, S. Clark, H. McNab. Preliminary report on the segregation of resistance in Chestnut to infestation by oriental Chestnut Gall Wasp. Acta Horticulturae 815 (2009) 33-35.

[7] G.Brussino, G. Bosio, M. Baudino, R. Giordano, F. Ramello, G. MelikaPericoloso insetto esotico per il castagno europeo. L'informatore Agrario 37 (2002) .

[8] C. Sartor, R. Botta, M.G. Mellano, G.L. Beccaro, G. Bounous, D. Torello Marinoni, A. Quacchia, A. Alma. Evaluation of susceptibility to *Dryocosmus kuriphilus Yasumatsu* (Hymenoptera: Cynipidae) in *Castanea sativa* Miller and in hybrid cultivars. Acta Hort (ISHS) 815(2009) 289–298 .

[9] Y. Murakami. A history of studies on the chestnut gall wasp in Japan. Proceedings, A Global Serious Pest of Chestnut Trees*, Dryocosmus kuriphilus:* Yesterday, Today and Tomorrow. (2010) 38–40. Japan.

[10] H.H. Flor. Host-parasite interaction in flax rust - its genetics and other implications. Phytopathology 45 (1955) 680–685.

[11] K.S. Mysore, C.M. Ryu. Non host resistance: how much do we know? Trends Plant Sci 9 (2004) 97–104.

[12] G.W. Fernandes. Hypersensitivity as a phenotypic basis of plant induced resistance against a galling insect (Diptera: Cecidomyiidae). Environmental Entomology, 27 (1998) 260-267.

[13] J.B. Harborne. Introduction to ecological biochemistry. London, UK: Academic Press (1988)

[14] J.L. Bi, G.W. Felton. Foliar oxidative stress and insect herbivory: Primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. Journal of chemical ecology. 21(10) (1995) 1511.

[15] C. Lamb , R.A. Dixon. The oxidative burst in plant disease resistance. Plant Physiology and Plant Molecular Biology, 48(1997) 251-275.

[16] M. Peng, J. Kuc. Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf disks. Phytopathology 82(1992)696-699.

[17] T. Ikegawa, S. Mayama, H. Nakayashiki ,H. Kato. Accumulation of diferulic acid during the hypersensitive response of oat leaves to *Puccinia coronata f. sp. avenae* and its role in the resistance of oat tissues to cell wall degrading enzymes. Physiol. Mol. Plant Pathol. 48 (1996) 245-255.

[18] R. Hückelhoven, R. Panstruga. Cell biology of the plant-powdery mildew interaction. Curr Opin Plant Biol 14 (2011) 738-746.

[19] A. Levine, R. Tenhaken, R. Dixon, C. Lamb. $H_2O_2$ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79 (1994) 583–593.

[20] Y. Tada, T. Mori, T. Shinogi, N. Yao, S. Takahashi, S. Betsuyaku, M. Sakamoto, P. Park, H. Nakayashiki, Y. Tosa, S. Mayama. Nitric oxide and reactive oxygen species do not elicit hypersensitive cell death but induce apoptosis in the adjacent cells during the defense response of oat. MPMI 17(3) (2004) 245–253.

[21] J.S. Bentur, M.B. Kalode, P.S. Prakash Rao. Reaction of rice (*Oryza sativa* ) varieties to different biotypes of rice gall midge (*Orseolia oryzae*). Ind J Agr Sci 64 (1994) 419-420.

[22] B.G. Lane, J.M. Dunwell, J.A. Ray, M.R. Schmitt, A.C. Cuming. Germin, a protein marker of early plant development, is an oxalate oxidase. J Biol Chem 268(1993)12239-12242.

[23] D. Patnaik, P. Khurana. Germins and germin like proteins: an overview. The Journal of Experimental Biology 39 (2001) 191–200.

[24] F. Bernier, A. Berna. Germins and germin-like proteins: plant do-all proteins. But what do they do exactly? Plant Physiology and Biochemistry, 39 (2011) 545–554.

[25] M. Delseny, P. Gaubier, G. Hull, J. Saez-Vasquez, P. Gallois, M. Raynal, R. Cooke, F. Grellet. Nuclear genes expressed during seed desiccation: Relationship with responses to stress. In Stress-lnduced Gene Expression in Plants, AS. Basra, ed (Chur, Switzerland: Harwood Academic Publishers) (1994) 25-60.

[26] B.G. Lane. Oxalate, germins, and higher-plant pathogens. International Union of Biochemistry and Molecular Biology Life 53(2002) 67–75.

[27] G.P. Bolwell, P. Wojtaszek. Mechanisms for the generation of reactive oxygen species in plant defence --a broad perspective. Physiological and Molecular Plant Pathology 51(1997) 347–366.

[28] H. Thordal-Christensen, Z. Zhang, Y. Wei, D.B. Collinge. Subcellular localisation of H2O2 in plants. $H_2O_2$ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant Journal 11 (1997) 1187-1194.

[29] M.T. Melillo, P. Leonetti, M. Bongiovanni, P. Castagnone-Sereno, T. Bleve-Zacheo. Modulation of reactive oxygen species activities and $H_2O_2$ accumulation during compatible and incompatible tomato- root-knot nematode interactions. New Phytol. 170 (2006) 501-512.

[30] N. Jambunathan. Determination and Detection of Reactive Oxygen Species (ROS), Lipid Peroxidation, and Electrolyte Leakage in Plants. Methods in Molecular Biology, 639(3) (2010) 291-297.

[31] Y. Gan, L. Zhang, Z. Zhang, S. Dong, J. Li, Y. Wang, X. Zheng. The LCB2 subunit of the sphingolip biosynthesis enzyme serine palmitoyltransferase can function as an attenuator of the hypersensitive response and Bax-induced cell death. New Phytologist 181(1) (2009)127–146.

[32] C. Sartor, D. Torello Marinoni, A. Quacchia, R. Botta. Quick detection of *Dryocosmus kuriphilus Yasumatsu (*Hymenoptera: Cynipidae) in chestnut dormant buds by nested PCR. Bulletin of Entomological Research, FirstView Article (2011) 1-5.

[33] J. Bogs, M.O. Downey, J.S. Harvey, A.R. Ashton, G.J. Tanner, S.P. Robinson. Proanthocyanidin Synthesis and Expression of Genes Encoding Leucoanthocyanidin Reductase and Anthocyanidin Reductase in Developing Grape Berries and Grapevine Leaves. Plant Physiology 139 (2) (2005) 652-663.

[34] R. Bronner. The role of nutritive cells in the nutrition of cynipids and cecidomyiidae. Biology of Insect Induced Galls (eds J.D. Shorthouse & O. Rohfritsch), Oxford University Press, New York. (1992) 118-140.

[35] G.N. Stone, J.M. Cook. The structure of cynipid oak galls: patterns in the evolution of an extended phenotype. Proceedings of the Royal Society of London Series B – Biological Sciences 265 (1998) 979 988.

[36] R. Botta, M.G. Mellano, G.L. Beccaro , G. Bounous. A. Alma, A. Quacchia, G. Bosio, L. Picciau. Chestnut gall wasp: preliminary results on biological control and evaluation of cultivar susceptibility [Castanea sativa Mill.; *Dryocosmus kuriphilus* Yasumatsu; Piedmont]. Italus Hortus 12(5) (2005) 29-30.

[37] G.W. Fernandes. Hypersensitivity: a neglected plant resistance mechanism against insect herbivores. Environmental Entomology 19(1990) 1173-1182.

[38] G.W. Fernandes, D. Negreiros. The occurrence and effectiveness of hypersensitive reaction against galling herbivores across host taxa. Ecological Entomology. 26 (1) (2001) 46–55.

[39] A. Barakat, D.S. DiLoreto, Y. Zhang, C. Smith, K. Baier, W.A. Powell, N. Wheeler, R. Sederoff, J.E. Carlson. Comparison of the transcriptomes of American chestnut (Castanea dentata) and Chinese chestnut (Castanea mollissima) in response to the chestnut blight infection. BMC Plant Biology 9 (2009) 51.

[40] A. Quacchia, C. Ferracini, J. Nicholls, E. Piazza, M.A. Saladini, F. Tota, G. Melika, A. Alma. Chalcid parasitoid community associated with the invading pest Dryocosmus kuriphilus in north-western Italy

.Insect Conservation and Diversity. (2012) 1-10.

## Legends Figures:

**Fig. 1:** Madonna buds. **A)** Bud at stage 1 after DAB treatment and boiling in Ethanol. **B)** Uninfected bud at stage 3 after DAB staining, the superficial scales were eliminated; the inner part appears whitish. Brownish staining, deriving from the reaction between hydrogen peroxide and diaminobenzidine, is not present. **C)** Infected open bud at stage 3, leaf primordia appear whitish after DAB staining. **D)** Infested bud at stage 1 shows cynipid eggs in the inner part. **E)** Infested bud at stage 3 exhibits larval cells in the last side of the internal leaf. **F)** Infested bud at stage 4 presenting a developing gall. **G)** Section of bud at stage 4 showing a larva into a gall.

**Fig. 2:** 'Bouche de Bétizac' buds. **A)** Bud at stage 1 after DAB treatment and boiling in Ethanol. **B)** Uninfested bud at stage 4 appears whitish in the internal scales after DAB staining. **C)** Infested bud at stage 1 containing cynipid eggs. **D)** Infested bud at stage 3 shows a cynipid larva into the larval cell just before the hypersensitive response. **E)** Infested bud at stage 4 presents a brown spot in the internal zone revealing DAB polymerization in presence of H2O2. **F)** Infested bud at stage 4 shows a larval cell in the left side undergoing HR response and a still developing larval cell at the right side. **G)** Brownish tissue found in the inner part of a larval cell in an infested bud at stage 4.

**Fig. 3**: RealTimePCR results showing GLP gene expression at the 2 different stage of budburst (2, 4) in 'Bouche de Bétizac' (B) and 'Madonna' (M). I=infested; N= not infested.

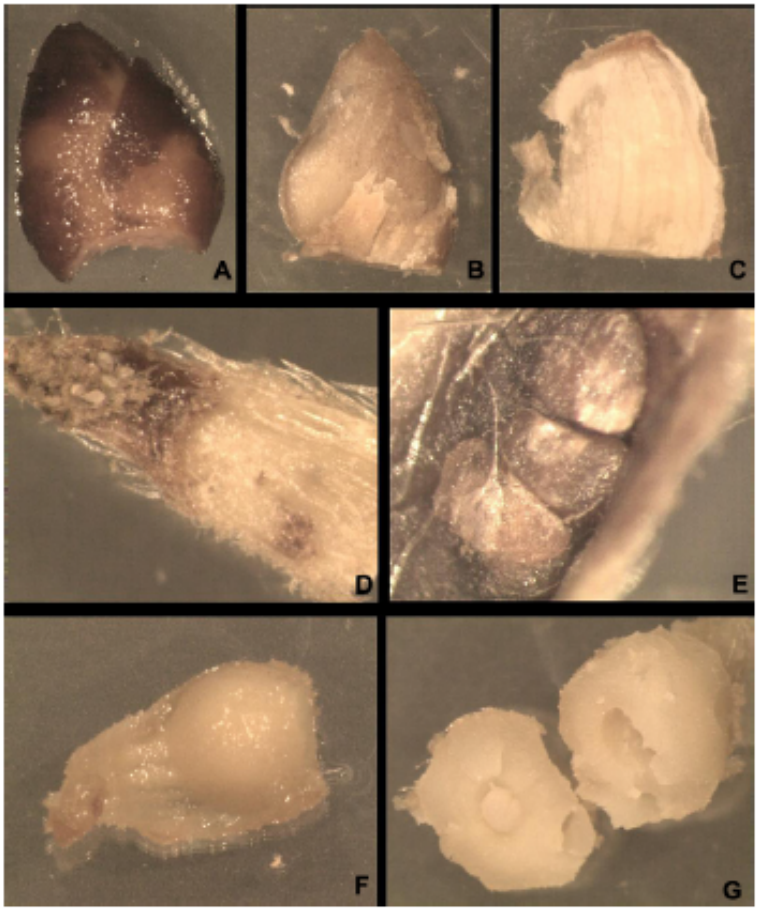


Fig. 1

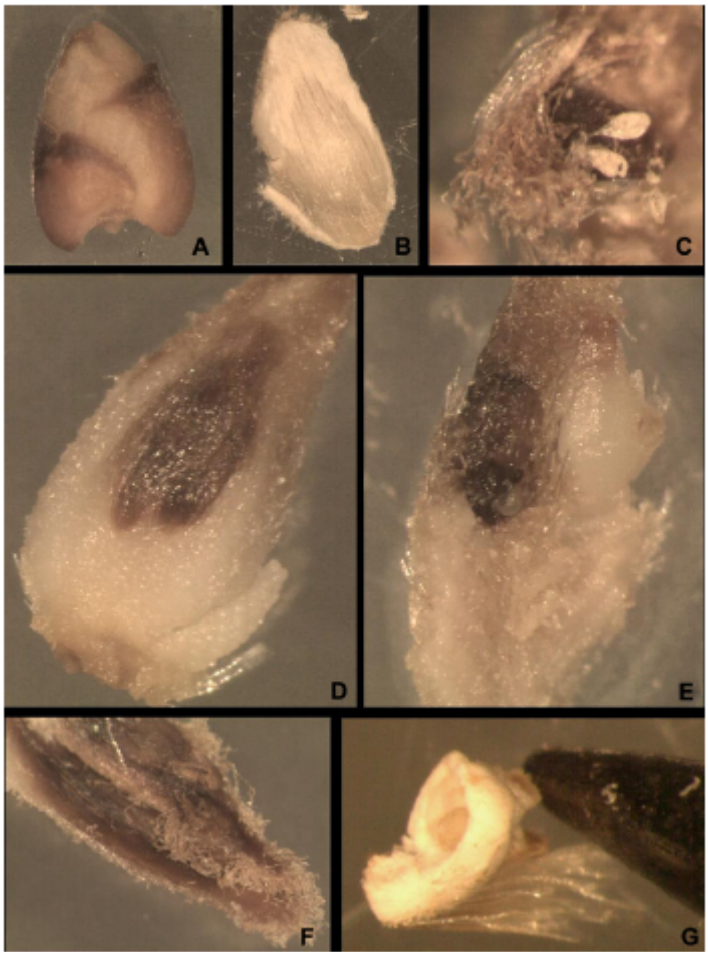
A
B
C
D
E
F
G

Fig 2

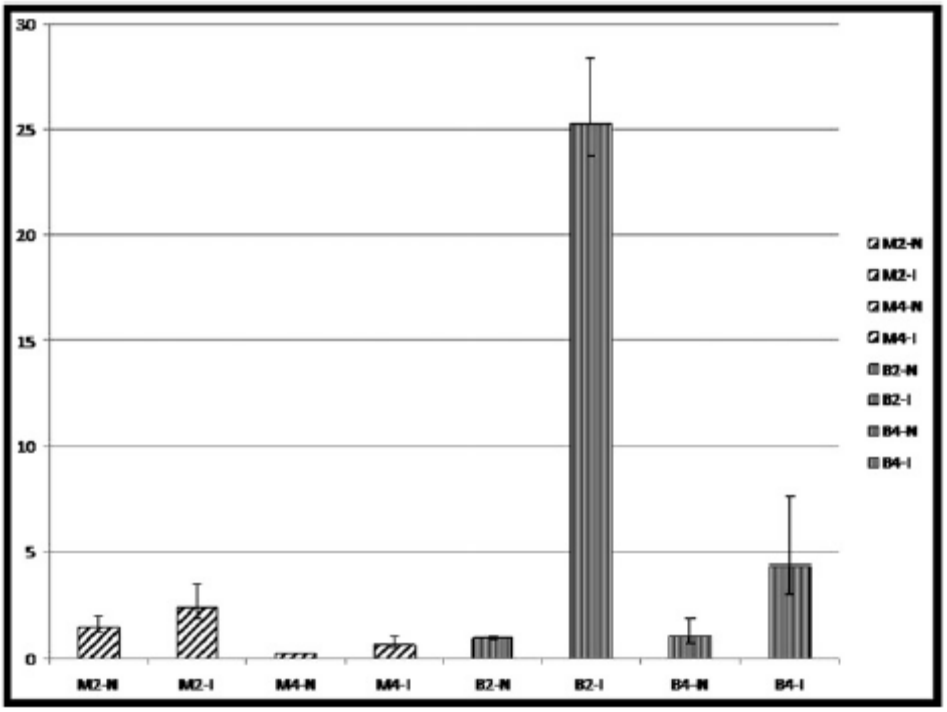
30
25
20
15
10
5
0
M2-N
M2-I
M4-N
M4-I
B2-N
B2-I
B4-N
B4-I
M2-N
M2-I
M4-N
M4-I
B2-N
B2-I
B4-N
B4-I

Fig 3