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Study of the biochemical and physiological dynamics occurring during grafting: a further step towards the prediction of graft incompatibility in chestnut

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Abstract:

The climate change is having dramatic consequences on agriculture because of the spread of new pests and diseases and the resulting abiotic stresses to which plants are subjected. It is essential to develop innovative cultivation strategies to adapt to the ongoing changes, such as the development of rootstock genotypes able to overcome these issues. In the last decades many chestnut hybrid rootstocks were selected for their resistance or low susceptibility to diseases, climatic conditions, and agronomic traits. Though, the main issue connected to their diffusion is the graft incompatibility. As grafting is the most used propagation technique on *Castanea* spp., it is essential to find techniques able to early predict this disaffinity, to drive breeding programs and therefore the development of the chestnut cultivation. Biochemical and physiological techniques have been tested in this regard with effective results on different fruit species. Phenol compounds seem to play a central role during grafting formation, as they are involved in cell division, development, and differentiation. Moreover, the physiological response of plants to graft-related stress have been monitored

through the study of stomatal conductance, fluorescence rate, and chlorophyll content. These quick and non-destructive parameters were found to be effective in the detection of differences among grafting combinations, standing out as supporting tools for biochemical analysis. The present work analysed the expression of phenol compounds and the physiological response to grafting in several chestnut combinations, testing different rootstocks. The identification and quantification of phenol molecules from vegetal samples was performed via HPLC, preceded by a double extraction with solvent and ultrasound-assisted extractor. Stomatal conductance, fluorescence, and chlorophyll of the leaves were measured during the vegetative cycle. The final goal was to find one or more techniques suitable to early predict grafting incompatibility, the main issue connected to chestnut breeding improvement. the combined study of these parameters contributed to acquiring greater insight into the graft incompatibility matter.

Keywords: Compatibility, clonal rootstocks, phenolic compounds, stomatal conductance, fluorescence

Protocols for the micropropagation of several chestnut varieties

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Abstract:

Materials of different chestnut varieties have been introduced and maintained in vitro in the Laboratory of Micropropagation of the Higher Polytechnic Engineering School of Lugo. Currently, stocks of the Galician varieties 'Negral', 'Branca', 'Amarelante', 'Longal' and 'Ventura', and of the Andalusian varieties 'Temprana' and 'Comisaria' are fully stabilized in vitro and preserved in in vitro conditions. This paper describes the general

conditions for their establishment, and several multiplication and rooting experiments.

The factors studied in the multiplication stage include, depending on the varieties, medium formulation (MS, WPM, GD), BA concentration (0 to 0.2 $mg \cdot L^{-1}$), and light regime (fluorescent lamps vs LEDs; colour temperature (4000, 5500, 6500 K)). In varieties that showed symptoms of endogenous bacterial contamination ('Longal' and 'Ventura'), the effect of supra-optimal concentrations of Cu (1µM -standard-, 30 µM, 60µM $CuSO_4$) was tested.

All the varieties were submitted to rooting induction (by dipping in IBA solution or in a medium with IBA), to test their rooting ability after stabilization of the cultures.

In general terms, all the varieties responded better, or with no significant differences, in multiplication rates, in WPM medium + 0.1 $mg \cdot L^{-1}$ BA. Regarding light regime, some varieties (e.g., 'Negral' and 'Branca') respond better under 4000 K (neutral) light, as compared to the standard 5500-6500K (cool-light). In general, multiplication rates under LEDs or fluorescent lights with the same colour temperature are similar for the varieties tested.

Concerning rooting ability, most of the varieties express low rooting percentages (0 to 25%), excepting 'Branca', which steadily shows rooting rates over 60%, and 'Negral' and 'Amarelante', which initially showed low rates, but improved significantly their rooting ability after 2 years of continuous subculturing. In the varieties showing endogenous bacterial contamination, 60 µM $CuSO_4$ was detrimental for the cultures. There were no differences in multiplication rates between cultures in 1 or 30 µM $CuSO_4$ after four successive subcultures, but the visual detection of bacteria around the base of the explants decreased drastically in medium with 30 µM $CuSO_4$.

Keywords: Andalusian varieties, Galician varieties, germplasm preservation, rooting

Development of a regeneration protocol for chestnut Italian cultivar ‘Marrone di Zocca’

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Abstract:

Among the different Italian varieties, *Castanea sativa* Mill. 'Marrone di Zocca' is remarkable for its high quality of nuts intended for the fresh market and industry transformation. Recently, this species has shown a particular susceptibility to several diseases determining a reduction in the cultivated area and a loss of production. To make this crop competitive again, it is essential to increase the investments to close the gap with the most important fruit crops. The optimization of an *in vitro* regeneration protocol from somatic tissues would allow the application of new breeding techniques (NBT) to this cultivar, reducing the time needed to obtain genetically improved and pathogen-free clones. This study is presented to propose a protocol for the regeneration of *C. sativa* cultivar 'Marrone di Zocca'. Leaf explants from micropropagated material, placed on various culture media suitable for regeneration, showed competence to shoot organogenesis, which we had already observed from in vivo-grow leaf tissues. The obtained regenerations, which show high proliferation rates once transferred to a suitable medium, will be further assessed for their possibility of increased rooting ability, to overcome one of the major bottlenecks of chestnut micropropagation.

Keywords: *Castanea sativa* Mill., in vitro, innovation, propagation, quality, NBT.

In Vitro Propagation of *Castanea sativa* Mill

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Abstract:

Castanea sativa Mill. is a versatile plant that has been extensively cultivated and highly appreciated for its economical value as foods, medicines, cosmetics, furniture, timber, etc. In southern Europe, Turkey, southwestern and eastern Asia, the species is considered as a staple food which made propagation and conservation are crucial for sustainability of the plant. Considering these facts, a great attention has been focused on various propagation techniques of the plant. In consequence, the present study attempted to focus on the ***in vitro*** propagation of ***Castanea sativa*** to meet the industry demand for elite and high quality of planting materials.This study aimed to obtain the optimum medium for production of the ***in vitro*** plantlets. Initially, ***Castanea sativa*** seeds were surface sterilized before subjected to MS basal for seed germination. Subsequently, the aseptic seedlings obtained were then excised and cultured in various treatments. After 2 months, observations were recorded on the percentage of explant producing shoots (%), mean number of shoots per explant, mean shoot height (cm), mean number of leaves and mean leaves length (cm). Results clarified the treatments tested mostly promoted 100% shoot production, and MS supplemented with CW + AC + 3.0 mg/L BAP + 1.0 mg/L NAA is the most recommended medium for ***in vitro*** regeneration. These findings contributed a fundamental understanding on propagation technique of ***Castanea sativa*** grown ***in vitro*** which significant in the production of planting materials for cultivation of the plant in Malaysia.

Keywords: Micropropagation, sweet chestnut, coconut water, activated charcoal, BAP and NAA.

The new EU chestnut nursery certification: processes, opportunities and critical issues for the sector

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Abstract:

The production of certified nursery material is a consolidated reality for most of the major fruit species, in particular pome and stone fruits. This paper presents the new opportunities and constraints for the chestnut nursery certification from the technical and legislative point of view.

In Italy there are 9 conservation and 10 pre-multiplication centres, located in 4 Regions (Veneto, Emilia-Romagna, Trentino-Alto Adige and Puglia), accredited for the production of certified plants.

The certification process includes genetic matching analysis and phytosanitary tests to verify the absence of viruses, fungi, phytoplasmas, bacteria and nematodes.

The main objectives of the genetic-sanitary certification are the production of materials in line with genetic and phytosanitary standards, the improvement of fruit quality and production, and the traceability of the supply chain.

The chestnut interest is today leading to a partial transformation from extensive to intensive chestnut cultivation and both the intensive and traditional chestnut orchards need the same operating standards of other fruit crops in order to be competitive.

The Chestnut R&D Center of Piemonte, recognized by the Italian Ministry of Agriculture as a Center for the conservation for the pre-multiplication and for the pre-multiplication of Italian chestnut cultivars (M.D., 11/20/2020), is landmark for certified plants production. The conservation and pre-multiplication phases for the production of basic plants, essential materials for the final production of certified chestnut groves, take place at the Center. The chestnut sector could finally rely on certified plants useful for an overall qualitative improvement. To be noted that among the varieties in conservation at the Center there are a selection of the principal local southern Italy varieties. Thanks to this selection made within the project

VALTIFRU 4.0, also the chestnut sector of the south of Italy could access to high-quality propagation materials.

Keywords: plant production, *Castanea* spp., traceability, quality

Efficient in vitro shoot proliferation of 'Marrone' accessions (*Castanea sativa* Mill.)

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Abstract:

In vitro propagation of European chestnut is hampered by several issues such as the heavy tannin exudation which drastically drops the efficiency of the culture establishment phase. This problem especially affects adult plant bud explants, which however must be used for selected cultivar propagation. Even once established *in vitro* on substrates so far reported as suitable for chestnut micropropagation the mature, somatic tissues of 'Marrone di Marradi' and 'Marrone di Zocca' show tannin leakage, poor reactivity and low proliferation rates. Notably, clorosis, hyperhydricity, tissue browning, early senescence and leader shoot tip necrosis prevent the yield of material vigorous enough to be used for further steps, such as rooting or grafting. Both cultivars 'Marrone di Zocca' and 'Marrone di Marradi' were proliferated on substrates with several salt and hormone compositions, either previously published, or newly tested on chestnut: among the latter, an alternative medium allowed a great improvement of both shoot proliferation and elongation. A consistent yield was obtained of large shoots, whose rooting ability or grafting compatibility with 'Marrone' seedlings are currently under evaluation.

Keywords: European chestnut, micropropagation, substrate, salt composition

Cryopreservation of Chestnut Zygotic Embryos by Dehydration 'One-Step Freezing'

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Abstract:

Georgia is the major part of the Caucasus; It is considered as one of the distinguished regions of the world with respect to biodiversity. The majority of Georgia's biodiversity is connected with forest ecosystems, which cover about 38% of the country's territories. Chestnut (*Castanea sativa* Mill.) is the dominant of mountainous forests of Western Georgia, which occupy the most percentage of areas covered with forests. Due to low self-renewal and diseases the big massifs of chestnut forests are on the verge of destruction. Cryopreservation at ultralow temperature (-196 °C) in liquid nitrogen is one of the most effective way for long-term conservation. In the present study, the efficacy of dehydration followed by 'one-step freezing' in liquid nitrogen of embryonic axes from mature chestnut seeds was evaluated. Survival and plantlet regrowth in post-cryopreservation of embryonic axes that underwent the dehydration/'one-step freezing' is discussed. Dehydration of chestnut mature zygotic embryos was carried out in laminar flow hood from 1 to 5 h, followed by direct immersion in liquid nitrogen. Dried embryos have been stored in liquid nitrogen for 24 hours. The viability, survival and regrowth of embryos were evaluated before and after freezing. TTC test was used for assessment of embryo survival after cryopreservation. Full germination of cryo-stored embryos after 5 h of dehydration (moisture content 21%) have been increased from 0% to 66.7%. All the plantlet from cryopreserved embryos could be easily acclimatized, producing healthy potted plants. Treatment of chestnut zygotic embryos with dehydration 'one-step freezing' technology allow the complete regeneration of the plantlets after freezing. We suppose that the current study will speed

up the protection of rare and endangered plant species providing efficient protocol for conservation of plant genetic resources.

Keywords: *Castanea sativa*, Dehydration, Cryopreservation, Zygotic embryos.

In vitro micrografting of selected Galician chestnut cultivars on chestnut hybrids (*Castanea x coudercii*)

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Abstract:

The recent certification in Spain of 23 varieties of Galician chestnut selected for their production of quality chestnut (Orden AAA / 605/2016, of April 20 and AMP / 848/2017, of August 30) and the high demand for grafted plants in Galicia make it necessary to develop new production strategies and deepen the knowledge of compatibility when grafting on Eurasian hybrids resistant to ink disease. This work offers first results of in vitro micrografting of chestnut varieties 'Ventura', 'Garrida', 'Negral', 'Amarelante' and 'Branca' on artificial hybrid rootstocks 3, 111, 7521 and 7810, in order to assess afinity and to evaluate the potential of the method to produce grafted plants. The initial results show that most of the combinations present high percentages of graft-taking, usually over 70%, and similar to those obtained by self-grafting. No external symptoms of incompatibility have been detected 2 to 4 months after graft-take.

Keywords: *Castanea sativa*, in vitro grafting affinity

In vitro establishment of chestnut clones of Martaínha variety

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Abstract:

Martaínha is a Portuguese chestnut cultivar widely appreciated for its excellent fruit quality, which stands out due to its pronounced sweetness, great calibre and good skinning. In this sense, Martaínha variety has been listed as a Protected Designation of Origin (PDO) “Castanha dos Soutos da Lapa”. Furthermore, as Martaínha is a precocious variety, disseminating this variety represents an exciting strategy to the sector, from an economic point of view. However, there has been a decline in Martaínha productivity caused by increasingly prolonged periods of drought, due to climate change. Consequently, nowadays, we face a growing fragility of trees, that are more sensitive and susceptible to pests and diseases. Additionally, many of the rootstocks used to graft Martaínha are not withstanding the stress induced by the biotic and abiotic conditions, leading to a massive tree loss. Therefore, it is imperative and emergent the identification of the most productive Martaínha trees with the best nut quality, to use tools that contribute to their conservation.
In this work, branches of selected Martaínha trees were collected to induce the sprouting of buds that were subsequently established in vitro, aiming at contributing to the preservation of this remarkable chestnut tree variety. In total, six material collections were carried out in the field between the end of October 2021 and the beginning of April 2022. On each monthly trip to the field, branches of 21-31 previously selected mother trees were collected, totalling 167 mother trees at the end of the semester. The shoots obtained from the collection carried out in December had a higher percentage of plant material that had passed to the multiplication phase, so this month seems to be the most suitable for carrying out future collections and thus guaranteeing

greater success in the establishment. *Acknowledgments*: work supported by project BreedMartainha (NORTE/01/0247/FEDER/072233) and by national funds provided by FCT to CITAB (project UIDB/04033/2020).