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Review Article

USE OF BIOSTIMULATING *SALIX* SOLUTIONS FOR THE VEGETATIVE PROPAGATION OF *CHRYSANTHEMUM*: A REVIEW

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Abstract: In the last decades, in horticulture, improving the propagation methods for decorative plants and raising their efficiency became a high priority. Preserving and maintaining the desired plants traits are essential and thus, vegetative reproduction *via* cuttings is usually the preferred way to obtain high quality material. Today, there are plenty of commercially available synthetic products that promise better and faster rooting and development of cuttings, but in most cases the price is very high and the environmental impact, due to their chemical composition, is another thing that must be taken into consideration. Finding new, eco-friendly and natural solutions that promote a better yield, which can be valued both economically and scientifically, determined research in the field and the results are promising. In this paper we aim to compile the present findings from relevant literature, still very scarce, underlining the importance of diversifying the solutions involved in increasing the natural growing and development of roots in plant cuttings, leading to a better percentage of rooting individuals with a minim impact towards the substrate in particular and the environment in general.

Keywords: *Chrysanthemum*, cuttings, natural extracts, rhizogenesis, willow extract.

Introduction

The chrysanthemum (*Chrysanthemum* s.l.) is one of the most important ornamental species, its production value increasing exponentially because of the fast improvement of the society's living conditions and lifestyle. The commercial varieties of chrysanthemum, especially the ones cultivated in pots, are usually vegetatively propagated *via* cuttings.

Chrysanthemum s.l. belongs to the Asteraceae (Compositae) family and is one of the economically important and favored floricultural crops, ranking second in the cut flower trade after roses [TEIXEIRA DA SILVA & al. 2020]. *Chrysanthemum* has a long history of cultivation; it was first cultivated in China as an herb since the 15th century BC and was then successively introduced in Japan, Europe, and the United States. The ancestry of modern chrysanthemum is still uncertain, but the plant is thought to have emerged mainly as a result of long-term artificial selection of variants belonging to several wild species, including *Chrysanthemum vestitum* (2n = 54), *C. indicum* (2n = 18, 36), *C. lavandulifolium* (2n = 18), *C. nankingense* (2n = 18), and *C. zawadskii* (2n = 54). Cultivated chrysanthemum is a complex hexaploid that also exhibits aneuploidy, in which chromosome numbers vary from 47 to 67 [DOWRICK, 1953; ROXAS & al. 1995]. However, a chromosome number of 54 is the most frequent and stable conformation (2n = 6x = 54) [CHEN & al. 1996; DAI & al. 1998; LIU & al. 2012].

In the last years, research was undertaken to accelerate the rhizogenesis process in many species that show difficulties in developing roots. This aspect mostly focused on using different chemical products (growing or rooting stimulators) to encourage rhizogenesis. The

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successful rooting of *Chrysanthemum* s.l. cuttings was reported under the influence of various factors, including type of cutting, rooting substrate, season, and environmental conditions, such as temperature, light and humidity.

In Romania, very few studies were conducted regarding alternative propagation methods and the conditions that affect the propagation in chrysanthemums [COJOCARIU & al. 2018; COJOCARIU & TĂNASE, 2019]. A part of these studies focuses on both the morpho-anatomical and biometric aspects, and on a series of modern approaches of *in vitro* multiplication of this species. After reviewing the relevant literature, we noticed that the research in the field of chrysanthemum cultivation was centered on the improvement of various flower traits, to increase their ornamental value. These characteristics include color, size and shape of the inflorescence and the quality of production. Although through classical sexual propagation methods the deserved results were obtained, there are some limits, such as restricted genetic diversity, limited cross breeding due to incompatibility and differences in polyploidy between the genitors, different flowering times and unequal growth. As a result, sexual multiplication can alter the balance between the factors that influence the plants' growth and development. A way to create and keep new varieties of plants is by using biotechnological methods. In micropropagation, the most common methods used for these purposes are the stimulation of axillary ramification and *in vitro* nodal sections cultivation [ROUT & al. 1996; ROUT & al. 2006].

The main methods of vegetative propagation through cuttings involves the stimulation of the plant's fragment tissue, using humidity and light, until the adventitious roots appear (AR) [OWEN & MAYNARD, 2007]. The AR development is a complex process that usually requires special environmental conditions and hormonal stimuli, which are specific to both species and tissue type [AGBO & OBI, 2007]. The modern propagation methods *via* cuttings use complex technologies, such as synthetic vegetal hormones, steam, and fine spraying systems, aeroponics, advanced hygiene systems and protocols to optimize the AR formation and to maximize the reproduction efficiency [PREECE, 2003].

Research history regarding the use of biostimulators in rhizogenesis

Plant biostimulators can be either microorganisms, microbial derivates or mineral, marine and vegetal materials that stimulate the physiological responses of plants, including induced systemic resistance (ISR) and improve nutrient and water absorption, promote plant growth and health, or offer tolerance towards biotic and abiotic stress factors, without having nutritive or phytosanitary properties. This definition covers both the technical and the regulatory aspects of biostimulators. However, there are some exceptions among the bio-stimulating products available on the market, because of the inherent nutritional and pesticide properties of some of their active ingredients [DARA, 2021].

YAKIN & al. (2017) offers a more complex definition, a synthesis of all the variants in the field and describes a biostimulator as a formulated product of biological origin that improves the plants' productivity as a result of the new and emergent properties of its ingredients and not as a unique consequence given by the presence of the plants' essential nutritional substances, growth regulators or plants' protection compounds.

In dicotyledonous plants in general, the AR can be defined as roots that can develop under specific conditions, from organs such as leaves or stems. AR formation in cuttings derived from stem tips with leaves is a crucial physiological process in the propagation of many ornamental plant species. Despite the intensive control over the environmental factors in the modern ornamental plants propagation industry, important economic losses still appear, due to an insufficient rooting [LIU & al. 2013].

Chrysanthemums (*Chrysanthemum* s.l.) have a significant ornamental value and a great economic importance, but their cultivation also involves losses caused by inadequate rooting of cuttings from young stems, which represents the main propagation method used for this culture. The insufficient understanding of the mechanisms that control the AR formation limits the use of efficient technologies for improving the AR development in ornamental plants' cuttings.

The adventitious rooting is a genetic trait and is influenced by many factors, both internal (endogenous), but also environmental (exogenous). One of the endogenous factors with a key role in the AR formation is the auxin. Numerous authors have proved that auxin has the capacity to initiate AR formation. PAGNUSSAT & al. (2002) showed that nitric oxide mediates the auxin response that leads to the development of AR in cucumber, and LIAO & al. (2010) studied the role of nitric oxide in the AR formation process in *Chrysanthemum*. The progressive accumulation and the local concentration of auxin at the base of the cuttings seems to play an important role in rooting initiation. [ACOSTA & al. 2009]. There are clearer studies that demonstrate that AR formation in many ornamental and industrial plant species also depends on the ethylene action [CLARK & al. 1999; SHIBUYA & al. 2004] that is produced following the injury of the plant tissue in cuttings making.

The plants' response to stress created by cutting the stem is a necessary step in the AR formation process [DA COSTA & al. 2013]. Once detached from the mother plant, the cutting must redistribute the remaining resources as fast as it can, to form AR and to reestablish the physiological balance, which allows the resource transfer in various parts of the cutting. Some studies showed that the distribution of carbohydrates in detached cuttings is probably more important than the intrinsic substance content [RUEDELL & al. 2013].

The origin of AR primordia varies anatomically and histologically depending on the species. In *Chrysanthemum* genus, for stem tip cuttings their formation is initiated in the interfascicular regions of the connection flanks of the pericyclic parenchyma [STANGLER, 1956]. The temperature at which the mother plants and cuttings are being kept influence the metabolism of AR. In the experiment done on *Camellia japonica* and *Chrysanthemum morifolium*, OOISHI & al. (1978) observed that the temperature effect on the cuttings can be mediated by carbohydrates' metabolization. The rooting took place in close connection with the temperature: 16%, 36% and 87% of rooted cuttings at 17 °C, 23 °C and 30 °C respectively. Chrysanthemums root earlier at 23 °C or 30 °C, but the root development doesn't continue, probably due to the different effects of the temperature on the primordia initiation and their following development. DYKEMAN (1976) tested the rooting of *Chrysanthemum* sp. and *Forsythia* sp. cuttings at 25 °C and 30 °C and observed a faster rooting and more roots forming at 30 °C, but the lengthening of the root, its diameter and the development of root hairs was superior at 25 °C. Another proven fact was that high temperatures favor the initiation of root primordia, while lower temperatures especially encourages the root development. The beneficial influence of higher temperature on the rhizogenesis initiation can be explained by the increase of respiration [OOISHI & al. 1978] and simple sugars catalysis, which are being stored at lower temperatures [HAISSING & al. 1986].

Considering the aspects of the root initiation and development stimulation, it is well known that AR formation in plants can be stimulated by the exogenous application of nitric oxide (NO) and hydrogen peroxide (H_2O_2), but the mechanism of this physiological response is still unclear. Research regarding the chrysanthemums were undertaken to understand the effects of NO and H_2O_2 on the cuttings rooting process and the biochemical changes inside the rooting area during rhizogenesis. The results showed that NO and H_2O_2 influence the rooting of chrysanthemums cuttings depending on their doses, with a maximum biological response at 50 μM sodium nitroprusside (SNP) as donor of NO and 200 μM H_2O_2. Also proven was a synergic effect between NO and H_2O_2 on the rooting mediation. NO and H_2O_2 treatments at adequate doses could increase the activity of polyphenol oxidase (PPO) and indoleacetic acid oxidase (IAAO) as well as the soluble carbohydrate content and total nitrogen, and at the same time decrease the total amount of polyphenols in the chrysanthemum cuttings. Moreover, the rooting percentage was significantly correlated with these activities or contents of the biochemical constituents. Together, these results indicated that NO and H_2O_2 treatments improved the AR development synergically and independently through the stimulation of PPO and IAAO activities and the carbohydrates and nitrogen content, simultaneously repressing the production of polyphenols [LIAO & al. 2010]. Similar biostimulating effects were observed for *Aloe vera* extract, including an accelerated plant and biomass growth, root development and oil production [EL SHERIF, 2017; HAMOUDA & al. 2012].

Research history regarding the use of *Salix* sp. extract

Salix genus includes mostly trees and sub-trees belonging to Salicaceae family, which comprises over 450 species, distributed all over the world and colonizing riparian habitats, including wet areas and water banks [ARGUS, 1997].

The willow bark extracts were extensively studied because of their medicinal implications and their role in plant-herbivores interactions. It was reported the presence of various secondary metabolites, such as salicin, triandrin, (+)-catechin, picein and salicortin, as well as polymeric tannins, but the content of these compounds varies a lot depending on the species, plant age, growth season and the solvent used for extraction [DOU & al. 2018]. The extraction with warm water represents a practical and cheap method to isolate the non-cellular substances from willow bark, having a significant yield, in a brief time. Time wise, it was proven that the extraction yield was high even after only 5 minutes, increasing up to 60 minutes and then remained relatively constant during long-time treatments. Raising the temperature from 60 to 80 °C led to a significant yield increase and further, raising the temperature up to 100 °C lowered the yield because of the partial degradation and/or extract precipitation at elevated temperature [DOU & al. 2018].

The willow extract was used for thousands of years in medicine for its analgesic effect and traditionally as a treatment for muscular osteoarthritic rheumatism, inflammation and pain, flu and respiratory problems, arthritis, gout, spondylitis, and rheumatoid arthritis, as well as for many other systemic diseases associated with inflammation [EMA, 2017]. Assyrians and Egyptians already knew the analgesic effects of a willow leaves decoct (extract) for relieving articular pain. At the beginning of the 19^{th} century, salicin (an alcoholic ß-glycoside of the salicylic acid) was extracted from the willow bark and purified: the active extract, named salicin was isolated in its pure form by Henri Leroux, a Franch pharmacist and by Raffaele Piria an Italian chemist, who managed to separate the acid in its pure form [LÉVESQUE & LAFONT, 2000]. Later, a Franch chemist, Charles Frédéric Gerhardt synthesized the acetylsalicylic acid from salicin, which became the active compound of the medicine called aspirin [FUSTER & SWEENY, 2011].

Many plant extracts are used especially as biopesticides, and less as biostimulators. Some products based on soybean and other plant oils are used both as biostimulators and biopesticides. However, there are few examples of vegetal extract that are used exclusively as biostimulators and indirectly contributed to the disease and pest control. Willow bark extract, which is used as a plant growth regulator and biostimulator, also shows fungicide properties [DENIAU & al. 2019].

Besides its use in medicine, many researchers tried to use willow bark extract in plant protection, because plant diseases represent a key problem in the economically important crops. KARST (2002) proved the efficacy of this extract in the protection of vineyards against vine downy mildew (*Plasmopara viticola*), and ARIF & al. (2009) also described its antifungal properties to explain this potential phytosanitary efficiency. For this purpose, the Ecological Institute for Food and Agriculture from France (ITAB) conducted the experimental field-testing program "4P" to align with the European Union (EU) general regulation regarding pesticides and reducing the use of copper. The "4P" program was initially designed to test plant extract as complementary solutions to overcome the use of copper in the horticultural and agricultural fields. Furthermore, a lot of work was put into homologating the willow bark extract as plant protection method by the EU. The willow bark extract was approved by the EU regulations regarding pesticides (EC Regulation 1107/2009) applicable in agriculture as base compound with antifungal properties [MARCHARD, 2016].

The *Salix* sp. bark extract is used to control foliar fungal diseases caused by *Taphrina deformans*, *Venturia inaequalis*, *Plasmopara viticola*, *Erysiphe necator* and *Podosphaera leucotricha*. Regarding the fruit trees (peach tree, apple tree) and vine (*Vitis vinifera*), several studies were conducted in France. A significant antifungal effect was observed when treatment with willow bark was applied (at a concentration of 220 g/ha). The effect of the aqueous extract is due to the high content of salicylic glycosides or salicylate that act by reducing the stress impact on the plant and by activating some defense mechanisms. However, the salicylic acid alone, compared to the willow extract, did not yield the same results. The *Salix* sp. bark decoct shown antifungal properties also by inhibiting spore germination [MARCHARD, 2015]. Many studies proved that salicylic acid has antifungal activity [Da ROCHA NETO & al. 2015; TOSUN & al. 2003].

The interest towards this compound as natural resource for plant protection is multiple; it is useful for plant protection acting as elicitor for defense and resistance mechanisms [DEMPSEY & KLESSIG, 2017]. This extract is also environmentally friendly, because the active molecules do not have biocide properties, being classified as safe for human consumption. The willow infusion inhibits the germination and spreading of fungal disease without killing the fungus, as the chemical pesticides do. Using the same recipe that is used in cultivating fruit trees and vine [MARCHARD, 2016], it was proven that *Salix* extract has antifungal activity towards *Botrytis cinerea* and *Penicillium expansum*, species that mainly contaminate post-harvest fruits [ANDREU & al. 2018; HUSSAIN & al. 2011].

MUTLU-DURAK & YILDIZKUTMAN (2021) demonstrated the potential of willow bark extracts (WBE) and willow leaves extracts (WLE) as vegetal biostimulators with key roles in improving the early growth in corn (*Zea mays*) in control and salinity induced stress conditions. In 3 days, the treatment of seeds with salicylic acid and willow extract increased the biomass of corn plantlets with 130% and 225%, respectively. The root surface was also improved by 43% with salicylic acid and with 87% with willow extract. Moreover, these extracts increased the protein concentration in leaves and reduced the negative effects of salinity during early growth. The decrease in lipids oxidation processes and the specific activity of antioxidant enzymes in willow extract treated seeds suggest a remission of the oxidative stress caused by salinity.

During saline stress conditions, it was proved that in corn leaves, the protein concentrations were improved by 50% to 80% when applying high doses of willow extract (leaves and bark). The plants treated with WLE presented a lower Na/K ratio compared to other treatments. So, the stress limiting effect of willow extracts can be partially attributed to the decrease in Na accumulation and to keeping a relatively low Na/K ratio. The results indicate the fact that willow can represent a valuable resource, and the leaves and bark aqueous extracts can be used as effective and eco-friendly biostimulators [MUTLU-DURAK & al. 2023].

Recent research proved that willow bark extract, which contains high amounts of salicylic acid (SA) as phytohormone, have biostimulating properties on plant growth, with known effects on flowering, callus formation, stress mediation and rooting. AL-AMAD & QRUNFLEH (2016) show that the percentage of callus formation in olive tree cuttings (*Olea europaea* L. 'Nabali') was lower (40.5%) under the effect of willow (*Salix babylonica*) leaves and bark extract compared to control (55.5%), probably due to the high quantities of cytokines accumulated in plant at the end of the vegetative season, while the bark and wood extract showed better results. The average number of formed roots/rooted cuttings was significantly higher in all experiments.

A series of studies demonstrated that SA and other salicylates in the willow extracts stimulate root growing in cuttings, seedlings and mature plants, in many species of economically important plants, such as: soybean (*Glycine max*) or carrot (*Daucus carota*) [BASU & al. 1969; GUTIÉRREZ-CORONADO & al. 1998; HAYAT & al. 2010].

KAWASE (1970) studied the presence of rooting stimulating compounds from young or lyophilized white willow (*Salix alba*) aqueous extracts. These molecules were split through paper chromatography or chemical fractionation, and their role in the rooting process was tested on mung beans (*Vigna radiata*) cuttings. GESTO & al. (1977) studied the content in rhizogenesis stimulating substances of two extracts: chestnut (*Castanea sativa*) and willow (*Salix viminalis*), proving that from September to June, the total phenolic content showed a similar model in both species, although for chestnut were observed two clear maximum peaks in February-March and June. Furthermore, in both extracts were identified compounds such as: p-hydroxybenzoic acid, p-coumaric acid, ferulic acid, gentisic acid, salicylic acid and cinnamic acid, while scopoletin and p-hydroxyphenylacetic acid were specific to the chestnut extracts, and caffeic acid, esculetin, catechol and saligenin were only found in willow extracts, (the last two compounds identified in large doses). Catechol proved to be efficient in increasing the number of roots and showed a synergic root growth effect with the indole-acetic acid (IAA). In the willow extract, IAA was present from April to June and contributed to a good rooting performance. IAA was identified exactly when the extract showed the highest rooting activity.

Research history regarding the use of *Salix* sp. extract in the vegetative propagation of chrysanthemums

The rhizogenesis biostimulating compounds represent an optimized method of vegetative propagation *via* cuttings, and the right application concentrations are, most of the time, species-specific. The use of willow extract as biostimulator on chrysanthemum cuttings was investigated especially for its capacity to stimulate AR formation and ramification, improving the propagation efficiency through cuttings in this species.

There are very few studies regarding the use of *Salix* sp. extracts for the rooting stimulation in chrysanthemums, although for other species the results were obvious and encouraging after using this solution.

Weeping willow (*Salix babylonica*) extracts contain a variety of bioactive compounds, including salicylates and phenolic compounds. Indole-3-butyric acid (IBA), present in willow extract, is a phytohormone which initiates and accelerates root formation. As a result of modern research, the rhizogensis stimulating effect of the commercial biostimulator Nutrifield Complex Root Nectar® (Nutrifield Pty Ltd., Melbourne, VIC) and willow bark extract were assessed on chrysanthemum (*Chrysanthemum* sp.) and lavender (*Lavandula × hybrida* 'Frills') cuttings. The commercial product applied at a concentration of 1 ml/L improved the rooting capacity in both species compared with the control, while a concentration of 1.06 µL/L of willow bark extract exceeded the results of the commercial product for both species [WISE & al. 2020]. The best results for chrysanthemum were observed when applying willow bark extract at a concentration of 1.06 µL/L, which led to root ramification in 13.9 days, while in the control cuttings the root ramification occurred after 18.4 days (12.83% faster in the case of willow bark extract). For lavender, all levels of willow extract treatment showed equivalent results compared with the control for both AR formation ($P<0.001$) and AR ramification ($P<0.001$). The AR biostimulating effects showed the willow bark extracts can be successfully used to accelerate the propagation in semi-woody species, underlining their applicability in increasing the process efficiency in the horticultural industry, dependent on fast vegetative propagation at a large scale [WISE & al. 2020].

Considering the fact that *Salix* stems produce an auxin named indole butyric acid (IBA), a hormone that naturally stimulates root growth, in the last few years (2022-2024) we conducted a series of studies within the chrysanthemum collection of "Anastasie Fătu" Botanical Garden of Iași – Romania, which aimed at testing the influence of a *Salix babylonica* water extract (SE) on the rooting process in *Chrysanthemum* s.l. cuttings.

The first step was represented by testing the SE in a controlled environment and establishing the optimal concentration for rooting stimulation. Within the second step several commercial products known for their rotting stimulating properties (Atonik, Clonex) were used to obtain comparative data related to the efficiency of *Salix* water extract [URSACHI, 2024]. The commercial rooting stimulating hormones are mainly made from synthetic forms of IBA. Alongside the rooting hormone, the willow stems also produce salicylic acid that are involved in wound healing, allowing the sap to circulate, giving roots the opportunity to develop. The aim of the research was to perfect the technology for obtaining chrysanthemum cuttings, improving the reproductive material yield, and raising the percentage of rooted cuttings, and thus the quality of planting material.

Based on direct visual observations and on measuring the newly formed AR, we were able to notice that the most efficient practical method is represented by treating the chrysanthemum cuttings with *Salix babylonica* extract at a concentration of 5%. An increase in SE concentration (10% or 20%) led to a decrease in root development, having mostly an inhibitor effect [URSACHI, 2024].

The new methods proposed after the research conducted within the "Anastasie Fătu" Botanical Garden chrysanthemum collection, following the testing of several *Salix babylonica* water extracts, aimed at raising the economic yield of the collections and can lead to a decrease of the total production costs, by using vegetal biostimulators, easy to obtain compared to the available commercial products. Testing the *Salix babylonica* extract in the experiments focused on obtaining viable cuttings on a standard multiplication substrate (peat + sand v/v) drove us to conclusions regarding its efficiency in rooting initiation and development, with results that can recommend this variant as a biostimulator treatment for cuttings rooting.

Conclusions

Chrysanthemums (*Chrysanthemum* s.l.) represent one of the major groups of ornamental plants that bring a significant contribution to the horticultural industry. To preserve the desired characteristics (general aspect, plant height, inflorescence color, size, shape, etc.) of the thousands of varieties created so far, the main propagation method used for this species is vegetative reproduction using cuttings. In practice, this classic method, optimized over time, still has some drawbacks, especially when referring to the cuttings capacity to form a powerful root system that can ensure the further development of the future plant.

Research in this field led to the commercial production of various chemical solutions that promise to resolve this issue, many of them with noticeable results. The two main problems of these products are the high production and selling price and their environmental impact, derived from their chemical composition. Because of these aspects, studies that test the biostimulating rooting capacity of natural products (especially vegetal extracts) started to be developed in the last few years, with encouraging outcomes.

One of the most promising plants in this direction is considered to be the willow (*Salix* sp.). Its extracts have been used for centuries in the medicinal and pharmaceutical industries for their analgesic effect, being known to treat muscular osteoarthritic rheumatism, inflammation and pain, flu and respiratory problems, arthritis, gout, spondylitis, rheumatoid arthritis, etc. A biochemical analysis of the willow extracts (bark, leaves, stems) showed the presence of many bioactive compounds such as salicin, triandrin, (+)-catechin, picein and salicortin, polymeric tannins, etc. which are used for their antifungal properties, but also for their capacity to promote plant growth, and to stimulate root formation and development.

Regarding the use of willow extracts as a biostimulator for roots formation in chrysanthemums, the studies are very few and relatively new, despite the promising results obtained for other economically important species, a fact that encouraged us to further investigate this aspect.

The outcomes of our research showed a positive influence of the willow bark extract on the rooting process in chrysanthemum cuttings, proving that a natural product, with no impact on the environment and cheap to produce can solve a problem that negatively affects the efficiency and propagation cost of this horticulturally significant species.

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