# Evaluation of *Agrobacterium tumefaciens* Usefulness for the Transformation of Sage (*Salvia officinalis L.*)

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Aleksandra Luwańska, Karolina Wielgus, Katarzyna Seidler-Łożykowska, Daniel Lipiński, and Ryszard Słomski

**Abstract**

Herbal plants have aroused people's interest for centuries as possible remedies for a number of conditions, in particular chronic and incurable diseases. Today, by combining genetic engineering methods with in vitro culture techniques, these plants can be modified for biomedical purposes as alternative systems for the production of biopharmaceuticals. The present research has examined possibilities of obtaining transgenic sage and aimed to establish a basis for preparing an oral, plant-produced recombinant vaccine against dental decay. Sage was chosen based on the fact that it is being used for treating oral cavity inflammations and in prophylaxis. The strain LBA4404 of *Agrobacterium tumefaciens*, most frequently used in the transformation of dicotyledons, was chosen for the transgenesis. The authors assessed the usefulness of this bacteria strain for sage transformation.

High antibacterial activity of sage against a wide range of microorganisms, including *A. tumefaciens*, may prevent or limit genetic modification in plants obtained by vector-based methods for transgenesis. Therefore, nonvector methods should be recommended to obtain the successful transformation of sage due to its strong antibacterial effectiveness.

**Keywords**

Sage • In vitro cultures • Transgenesis • *Agrobacterium tumefaciens* • Antibacterial activity • Secondary metabolites

A. Luwańska (✉) • K. Wielgus (✉) • K. Seidler-Łożykowska (✉)
Institute of Natural Fibres and Medicinal Plants, Poznan, Poland
e-mail: aleksandra.luwanska@iwnirz.pl; karolina.wielgus@iwnirz.pl; katarzyna.lozykowska@iwnirz.pl

D. Lipiński (✉) • R. Słomski (✉)
Department of Biochemistry and Biotechnology, Poznan University of Life Sciences, Poznan, Poland
e-mail: lipinskidaniel71@gmail.com; slomski@up.poznan.pl



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## Contents



# 1 Introduction

## 1.1 Botanical Description of Sage

*Salvia* genus belongs to the Nepetoideae subfamily in the Lamiaceae family. About 900 species of the plant have been recognized [1]. Sage (*Salvia officinalis* L.), a small evergreen plant, originating from the Mediterranean and Asia Minor, has been used for ages as a medicinal plant [2]. Sage is a perennial dwarf shrub. It grows up to 40–80 cm in height and has silvery-green lance-shaped leaves covered with tomentose. The plant blooms in the second year of cultivation, and its violet flowers are attractive to insects. The fruit is a schizocarp which splits into four black smooth-surfaced spherical mericarps [3]. The weight of 1000 seeds is approximately 8 g. Seeds are used to establish sage plantations. Sage needs rich, permeable, moist soil. The plants are resistant to drought. In Poland, however, they can be sensitive to frost, particularly during snowless winters. A perfect site for growing sage should be warm, isolated, and protected against winds. In crop rotation, sage can be cultivated after rape, root crops, or legumes. Sage is not a good forecrop as it leaves the field dried and weedy. Sage is cultivated in Europe, Russia, North America, and Africa. In Poland it is cultivated on an area of 600 ha. The Polish sage cultivar "Bona" produces the yield of 3 $t \cdot ha^{-1}$ of dry herb, containing 1.4% of essential oils. As 95% of flowers are male fertile, the seed yield is approximately 0.5 $t \cdot ha^{-1}$.

## 1.2 Sage Secondary Metabolites and Their Activity

Sage leaves (*Salviae folium*) and herb (*Salviae herba*) are raw materials harvested before flowering. Sage raw material contains essential oil, tannins, flavonoids, saponins, resins, and vitamins. The major components of sage oil are monoterpenes: α- and β-thujone, camphor, borneol, and 1,8-cineole [4–7]. The precursors of all sage

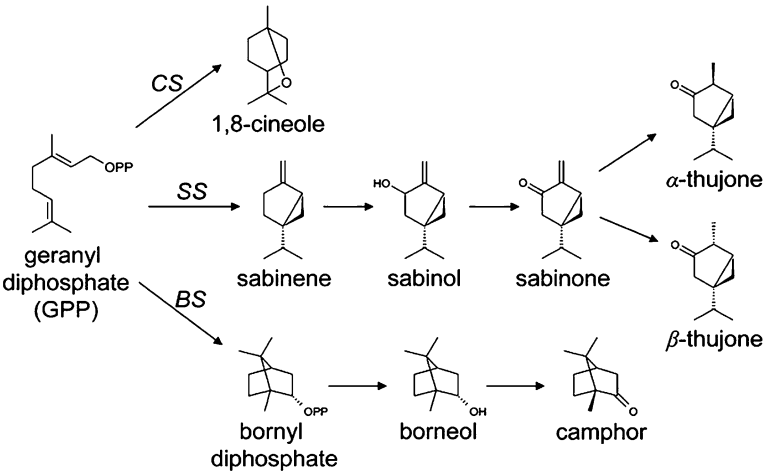


**Fig. 1** Three terpene synthases and their products: 1,8-cineole synthase (*CS*) for the one-step formation of 1,8-cineole, (+)-sabinene synthase (*SS*) as a first step to α- and β-thujone, and (+)-bornyl diphosphate synthase (BS) en route to borneol and camphor (based on the data of Grausgruber-Gröger et al. [7])

monoterpenes are geranyl diphosphate (GGPP) and neryl diphosphate formed in the plastids via the 1-deoxy-D-xylulose-5-phosphate pathway [7]. Three distinct monoterpene synthases are responsible for the first steps in the formation of the major monoterpenes (Fig. 1). The (+)-sabinene synthase (SS) catalyzes the production of sabinene, which undergoes further rearrangements leading to the two major monoterpenes α- and β-thujone. The 1,8-cineole synthase (CS) produces in one step 1,8-cineole. Finally, (+)-bornyl diphosphate synthase (BS) produces bornyl diphosphate, which is subsequently hydrolyzed to borneol and then oxidized to camphor.

Monoterpenes are produced and accumulated mainly in epidermal glands. After their formation in the metabolically highly active secretory cells, they are transported through the cytoplasm and the cell wall and stored in subcuticular oil storage cavities. Grausgruber-Gröger et al. [7] investigated the seasonal influence on the formation of the main monoterpenes in young leaves of the field-grown sage plants in two cultivars at the level of mRNA expression, analyzed by qRT-PCR, and at the level of end products, analyzed by gas chromatography. All monoterpene synthases and monoterpenes were significantly influenced by cultivar and season. 1,8-Cineole synthase and its end product 1,8-cineole remained constant until August and then slightly decreased. The thujones increased steadily during the vegetative period. The transcript level of their corresponding terpene synthase, however, showed its maximum in the middle of the vegetative period and declined afterwards. Camphor remained constant until August and then declined, exactly correlated with the mRNA level of the corresponding terpene synthase.

Sage raw material also contains catechin; tannins; triterpenes; flavonoids; bitters (carnosol); organic acids; vitamins B1, C, and PP; and carotenes [8–11]. In many studies, sage is mentioned as a source of polyphenol compounds. Rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid, was identified as one of the active components with high biological activity. The phenylpropanoid and tyrosine-derived pathways are both involved in the biosynthesis of rosmarinic acid in plants (Fig. 2). L-phenylalanine (L-Phe) is transformed to 4-coumaroyl-CoA by the enzymes of the phenylpropanoid pathway (phenylalanine ammonia lyase, cinnamic acid 4-hydroxylase, and hydroxycinnamic acid: coenzyme A ligase). On the other side, tyrosine can be transaminated to 4-hydroxyphenyllactate (pHPL) by two enzymes: tyrosine aminotransferase and hydroxyphenylpyruvate reductase. The hydroxycinnamoyl moiety of hydroxycinnamoyl-CoA is then transferred to the aliphatic hydroxyl group of a hydroxyphenyllactate by hydroxycinnamoyl-CoA: hydroxyphenyllactate hydroxycinnamoyltransferase ("rosmarinic acid synthase," RAS) which was shown to prefer the monohydroxylated substrates, 4-coumaroyl-CoA and pHPL. The hydroxyl groups in positions 3 and 3′ of the aromatic rings are finally introduced by cytochrome P450 monooxygenases [11].

Sage is known for its multiple pharmacological effects including antibacterial, antiviral, antioxidant, anti-inflammatory, antidiabetic, and antitumor properties, which are related to its active compounds [7, 11–18]. Sage essential oil has a strong inhibiting effect against the multiplication of numerous species of gram-positive and gram-negative bacteria, including those resistant to antibiotics. The oil also neutralizes bacterial toxins and has an antioxidant effect [19, 20]. The antiseptic effect of the oil is used as an alternative method for controlling microorganism multiplication and for food preservation [21, 22].

Sage extracts are used in treating gastrointestinal inflammations, bleeding, and ulcers [23]. Sage is also recommended for rinsing inflamed mucous membranes in the oral cavity and throat [24]. It is also used in excessive intestinal fermentation, flatulence, gastritis, and diarrhea as it regulates the functioning of the digestive system.

Sage raw material is an ingredient of numerous medications and medicinal preparations. What is more, in vitro research has demonstrated that sage is highly active against the Herpes simplex viruses HSV1 and HSV2 [24]. The chemical compounds in the raw materials carnosol, epirosemanol, and methyl carnosol play an important role in the prophylaxis of numerous diseases of affluence, such as atherosclerosis, diabetes, cataract, liver diseases, Parkinson disease, and Alzheimer disease [25–27]. Sage extracts protect cells from DNA damage, stimulate its repair [28], and inhibit the development of cancer cells [29]. In recent years it has been discovered that diterpenes isolated from sage activate PPARs, which influences their antidiabetic effect [30]. Sage preparations are also used in treating menopause symptoms [31]. Sage is also used as herbal tea and spice and in cosmetics and perfumery [32].

High thujone content in the essential oil can produce negative effect of the raw material. The EU Scientific Committee on Food [33] indicates that α-thujone acts as a GABA blocker and is three times more potent than β-thujone and that thujone

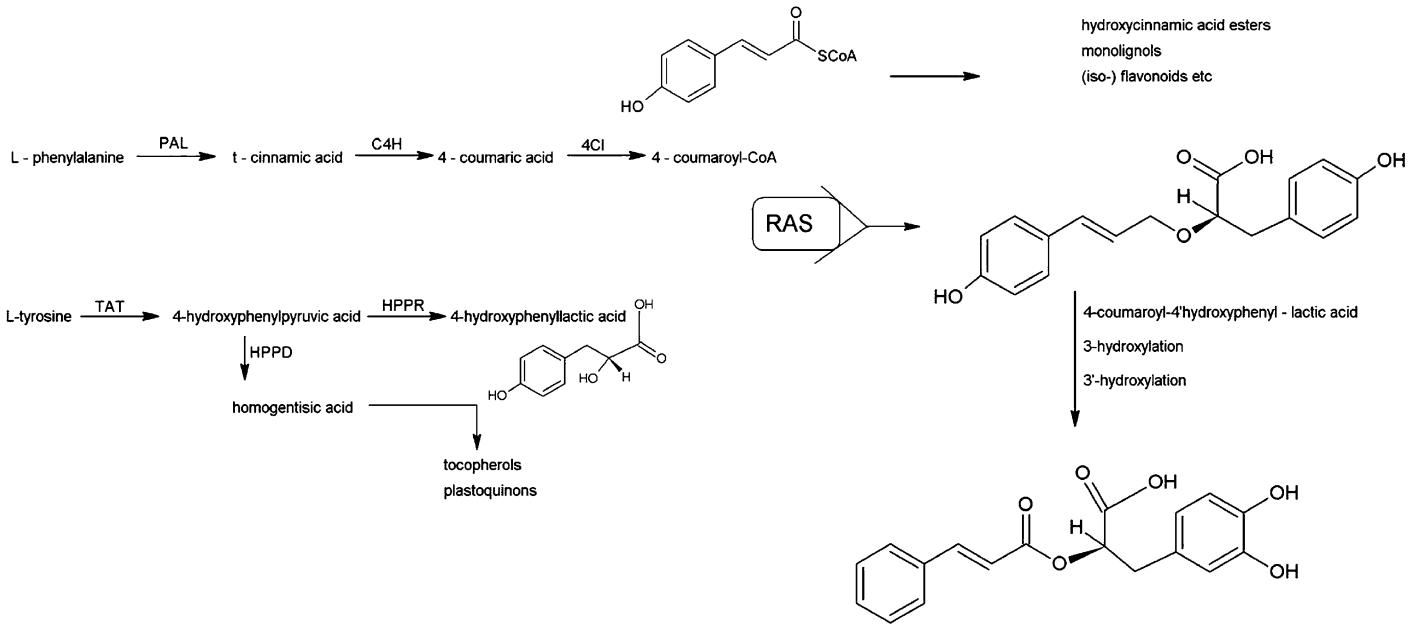


**Fig. 2** The biosynthetic pathway leading to rosmarinic acid with some side reactions. PAL phenylalanine ammonia lyase, C4H cinnamic acid 4-hydroxylase, 4CL 4-coumarate:CoAligase, TAT tyrosine aminotransferase, HPPR hydroxyphenylpyruvate reductase, HPPD hydroxyphenylpyruvate dioxygenase, and RAS rosmarinic acid synthase (based on data of Ejtahed et al. [11])

causes convulsions and epileptiform seizures. The maximum sufficiently protective daily dose of thujone for humans is 7 mg [33]. What is more, there have been reports on toxic effects of sage on newborns and infants [34].

## 1.3 In Vitro Production of Sage Secondary Metabolites

Herbal raw material derived from in vitro cultures is gaining popularity as an alternative method of obtaining secondary metabolites for multipurpose use. The fact that in vitro cultures allow for free control of the process may have a significant effect on the increase in secondary metabolite synthesis [35]. The development of biotechnological methods for producing standardized valuable phytochemicals (e.g., food additives, pharmaceuticals, and pesticides) based on plant in vitro cultures offers the advantages of continuous production under controlled conditions, independently of environmental factors (geographical latitude, climatic change, and seasonal variation) [36]. The potential of in vitro *Salvia* systems as tools for controlled production of valuable secondary metabolites has been investigated [18]. In particular, considerable attention has been paid to *S. miltiorrhiza*, although the content of *S. officinalis* active substances from in vitro system was also reported (Table 1). The more recent findings confirmed usefulness of cell suspensions and hair root cultures as the potential of terpenoids and polyphenols accumulation.

Only a few reports have described cell suspensions and hairy roots system from sage as a source of secondary metabolites. In a comparative study of various sage in vitro systems with different levels of differentiation, it was found that hairy roots and cell suspensions were the best systems for rosmarinic acid production, whereas the shoot cultures were the only system that produced carnosic acid and carnosol [19]. Bolta et al. [39] observed that the biosynthesis of ursolic acid by sage cell culture was adversely affected by cell differentiation: the single-cell fraction of sage cell suspension accumulated almost 50-fold higher amount of ursolic acid than highly aggregated suspension culture. However, the tested sage cell suspensions exhibited negligible biomass accumulation when forced to grow as single cells [39]. Hairy root cultures are the most effective in vitro systems for the production of valuable secondary metabolites whose biosynthesis occurs primarily in the roots of mature plants [60]. The modified metabolism of sage hairy roots may also result in production of novel biologically active compounds that are not typically found in the field-growing plants [18]. Genetic engineering, elicitation, [51, 54, 59] and even bioreactor designs may improve the yields of produced secondary metabolites of sage hairy roots. Grzegorczyk and Wysokińska [70] reported that sage hairy roots accumulated up to 1.6-fold more biomass when cultivated in nutrient sprinkle bioreactor in comparison with shake-flasks cultivation. They also found that the amount of accumulated rosmarinic acid from this bioreactor system was up to fivefold higher than that detected in mature sage plants. Also Grzegorczyk et al. [19] found that hairy roots of sage accumulated higher amount of rosmarinic acid (31 mg rosmarinic acid/g DW) than mature plant roots, in vitro shoots, or cell suspension cultures, and therefore their extracts exhibited higher antioxidant activity.

**Table 1** Recently reported bioactive terpenoids and polyphenols from *Salvia* in vitro systems (based on the data of Marchev et al. [18])

| Secondary metabolite | Species/in vitro system | References |
|---|---|---|
| Triterpenes | *S. tomentosa*/ callus; *S. scabiosifolia*/ callus; *S. officinalis*/cell suspension; *S. officinalis*/ shoot culture; *S. sclarea*/hairy roots; *S. austriaca*/ hairy roots | Georgiev et al. [37]; Marchev et al. [38]; Bolta et al. [39]; Grzegorczyk et al. [19]; Kuźma et al. [40]; Kuźma et al. [41]; Kuźma et al. [42] |
| Tanshinones | *S. miltiorrhiza*/ cell suspension; *S. miltiorrhiza*/ hairy roots | Wu and Shi [43]; Yuan et al. [44]; Zhao et al. [45], (2011) [46]; Gupta et al. [47]; Yan et al. [48]; Yang et al. [49], [50], [51]; Kai et al. [52], [53]; Gu et al. [54]; Liang et al. [55] |
| Hydrophilic phenolic compounds | *S. miltiorrhiza*/ hairy roots; *S. miltiorrhiza*/ cell suspension | Yuan et al. [44]; Xiao et al. [56]; Dong et al. [57]; Gu et al. [54]; Hao et al. [58] |
| Phenolic acids | *S. officinalis*/ hairy roots; *S. officinalis*/cell suspension; *S. miltiorrhiza*/ hairy roots *S. miltiorrhiza*/ cell suspension *S. tomentosa*/ hairy roots | Grzegorczyk et al. [19]; Xiao et al. [56]; Dong et al. [57]; Zhao et al. [46]; Xiao et al. [59]; Gu et al. [54] |

Sage cell suspension cultures currently seem to be more useful as model systems for biochemical, biosynthetic, and genetic studies. However, it should be considered as a potential method for the commercial production of sage secondary metabolites. Sage is also an attractive subject for hairy roots induction, mainly because of its potential to produce a wide range of biologically active compounds that accumulate in the roots [18].

## 2 Estimation of Sage Secondary Metabolites in In Vitro Cultures

In vitro culture conditions (the type and concentration of plant growth regulators) are different for every species. Micropropagation conditions for species of the *Salvia* genus, such as *Salvia sclarea* [61], *Salvia fruticosa* [62], and *Salvia guaranitica* [63],

have been established. In the Institute of Natural Fibres and Medicinal Plants (INF&MP), an effective protocol for the micropropagation of sage cultivar "Bona," which ensures a rapid growth of the plants, has been developed [64]. Effective micropropagation and successful regeneration of the plants were achieved by using cytokinins: 6-benzylaminopurine (BA) or meta-topolin (mT) in the concentration of 0.3 mg/L in apical and axillary bud cultures.

In the 2-week cyclic culture period, the multiplication rate (MR) was similar in all combinations, except for the MS BA + NAA combination, where it was significantly lower (Table 2). Cultures grown on media containing BA or mT were characterized by a good condition of explants (creating buds and growing) during cyclic micropropagation, as well as high multiplication rate. Other combinations of growth regulators decreased the multiplication rate, and the explants were characterized by a worse physiological condition (vitrificated and necrosed). In her research, Gostin used BA during a 6-week sage culture, obtaining an effective regeneration of plants with good morphological parameters [65]. The author also obtained regenerated morphologically normal sage plants by using kinetin (KIN) together with 1-naphthaleneacetic acid (NAA). The usefulness of BA was documented together with other growth regulators: 2,4-dichlorophenoxyacetic acid (2,4-D) [20] or indole-3-acetic acid (IAA) [66]. The innovative use of mT, which had not been used in sage micropropagation before, had a positive effect on the proliferation of buds and may become an alternative for the widely used BA cytokinin.

As far as biosynthetic properties are concerned, callus and suspension cultures are particularly useful as a possible valuable source of secondary metabolites for industrial use. However, in the case of unorganized cultures, there may be differences in the composition of secondary metabolites as opposed to organized cultures. Analysis of sage chemical composition showed that the production of diterpenoid compounds (carnosic acid and carnosol) is closely linked to the differentiation of organs. As regards the production of rosmarinic acid, it was similar in callus and suspension cultures and in in vitro and in vivo grown plants [67]. Callus cultures and

**Table 2** Selected plant growth regulators used in micropropagation of apical and axillary buds

| Combination | | Multiplication rate (MR) | Explants [%] | | | |
|---|---|---|---|---|---|---|
| | | | Creating buds | Growing | Vitrificated | Necrosed |
| Medium | MS 0.3 BA | **2.952a** | **91.1a** | **77.5a** | **5.5c** | **3.19bc** |
| | MS 0.3 mT | **2.732a** | **98.6a** | **93.9a** | **4.8c** | **1.14c** |
| | MS BA + IAA | 2.550a | 96.3a | 86.8a | 12.4bc | 1.69c |
| | MS BA + NAA | 1.847b | 59.8c | 36.7b | 24.8b | 38.48a |
| | MS 0.3 BA | 3.095a | 83.2b | 33.7b | 54.8a | 11.58b |
| $L.S.D_{0,05}$ | | 0.6843 | 15.28 | 22.14 | 14.83 | 8.564 |

suspension cultures [68, 69] as well as liquid bud cultures of sage may be a valuable source of phenolic acids used for controlled production of antioxidant substances in bioreactors [70].

The composition of certain active ingredients of the raw material obtained from apical and axillary bud cultures may vary due to somaclonal variation in plants multiplied in in vitro cultures [71–73].

In the present study, phytochemical analysis was conducted to compare sage raw material derived from field cultivation (in vivo) with in vitro cultures (Table 3).

The HPLC analysis and spectrophotometrical measurement showed an increase in polyphenolic compounds and polyphenol acids in plant material derived from in vitro culture and a decrease in flavonoids compared to the raw material derived from field cultivation. The increase in polyphenolic compounds and the decrease in flavonoids may be related to the physiological reaction of the plants grown in in vitro cultures caused by infection- and damage-related stress.

Correlation analysis of features showed an inversely proportional relation between the flavonoid content per quercetin and the content of polyphenolic compounds. Negative effect of drying on the flavonoid content per hyperoside was also noted in in vitro and in vivo cultures (Table 4).

Negative correlation between the content of flavonoids and polyphenolic compounds in the raw material derived from in vitro cultures may result from metabolic changes caused by increased stress. Lower intensity of light used in in vitro cultures as compared to in vivo cultures was an important stress factor for the plants. It might have limited the biosynthesis of flavonoids, increasing the activity of other phenol compounds with antioxidant properties [74].

The antioxidant properties of sage are determined by the synergistic effect of various phenolic compounds [75] or the presence of rosmarinic and carnosic acids [76]. The observed decrease in the amount of flavonoids and increased biosynthesis of other polyphenolic compounds (rosmarinic acid, other polyphenol acids) may have stemmed from triggering certain metabolic pathways at the cost of others under stress conditions in in vitro cultures. As regards polyphenol acids, which participate in the synthesis of lignins responsible for the stiffening of cell walls [77], their increased content limited plant growth in in vitro cultures as compared to field-cultivated plants. The amounts of tannins, which inactivate enzyme proteins in necrotic tissues, remained unchanged regardless of growing conditions.

## 3 Sage Genetic Transformation

The wide range of possibilities offered by secondary metabolites obtained from sage in vitro cultures triggered the search for new methods of improving *Salvia officinalis* through genetic transformation. The micropropagation protocol for sage cultivar "Bona" enabled for obtaining and storing massive amounts of selected plants with the best medicinal parameters for genetic modification. Few publications are to be found in literature regarding the transformation of sage. Researchers have investigated the use of *Agrobacterium rhizogenes* for obtaining transformed sage roots for

**Table 3** Secondary metabolites content in sage leaves

| Growing conditions | Content [%] | | | | | Loss of weight after drying [%] |
|---|---|---|---|---|---|---|
| | Flavonoids per | | Tannins per pyrogallol | Polyphenolic compounds per rosmarinic acid | Polyphenol acids per chlorogenic acid | |
| | Perquercetin | Perhyperoside | | | | |
| In vivo | 0.52 | 0.74 | 1.52 | 4.07 | 4.33 | 6.53 |
| In vitro | 0.37 | 0.53 | 1.45 | 6.37 | 7.38 | 6.46 |

**Table 4** Correlation coefficient of sage secondary metabolites obtained in in vitro and in vivo conditions

| Feature | Flavonoids per quercetin | Flavonoids per hyperoside | Tannins | Polyphenolic compounds | Loss of weight after drying |
|---|---|---|---|---|---|
| Flavonoids per quercetin | – | | | | |
| Flavonoids per hyperoside | 0.5992 | – | | | |
| Tannins | −0.3301 | 0.2925 | – | | |
| Polyphenolic compounds | −0.9815*** | −0.5274 | 0.3474 | – | |
| Loss of weight after drying | −0.4806 | −0.9139* | −0.2883 | 0.3782 | – |

Correlation significance * $p < 0.05$; *** $p < 0.001$

the production of rosmarinic acid [78] and other species of sage: *S. sclarea*, *S. miltiorrhiza*, and *S. przewalskii* [79]. There is no data, however, on developing sage transformation procedures using *A. tumefaciens* or nonvector-based methods.

The aim of presented research was to investigate the possibilities for obtaining a transgenic variety of the sage and creating a basis for an oral, plant-produced recombinant vaccine against dental decay. The main research goal was to obtain transgenic sage containing a gene coding for the surface protein antigen I/II in *Streptococcus mutans*, the main factor responsible for the development of dental decay in humans. The antigen could be a valuable component of a vaccine against dental decay as it causes a relevant immunological response. Dental decay is considered a socially determined disease, with *Streptococcus mutans* as the primary pathogen in the etiology of the disease. Obtaining a transgenic sage mutation would be a good basis for developing a preparation based on the medicinal properties of the active ingredients found in the plant and the immunogenic properties of certain *S. mutans* proteins, without the need for their costly extraction and purification.

Prior to commencing the process of transformation, the concentration of the selection agent (kanamycin antibiotic), lethal to non-transgenic plants, was established. The transformed cells containing genes coding for the enzyme which decomposed the antibiotic survived the selection. Performing the selection was the easiest way to control the transformation process. However, antibiotic selection is currently avoided with plants grown for consumption [73]. Selectable markers determining the identification of transformants remain in the plant genome even after they have fulfilled their function and may affect microorganisms living in a given environment. Moreover, the presence of selectable markers may be an obstacle for subsequent transformations. The idea of the research excluded the danger of horizontal gene transfer in the cultivation, allowing for this kind of selection. Recent developments open new directions in research, aiming at eliminating or silencing selectable markers in genetically modified plants [80].

### 3.1 Methods of Transformant Selection

In order to establish the appropriate concentration of kanamycin, experiments on apical and axillary buds obtained both from seedlings and multiplants were conducted. The MS medium with 0.3 mg/L BA was used. Selectable concentrations for seedlings and multiplants were established by using six variants of kanamycin concentrations (0, 25, 50, 100, 150, 200 mg/L), with 100 explants in each combination in two replicates. The analyzed kanamycin concentrations affected the longevity of explants in a variety of ways (Fig. 3). Lethal kanamycin concentration for sage explants both from seedlings and multiplants was established at 200 mg/L (Fig.3f).

### 3.2 Methods of Plant Transformation

Prior to begin the process of sage transformation, *A. tumefaciens* LBA4404 strain was prepared according to the transformation protocol in the following stages:

(a) Frozen bacterial cells containing CRSA gene construct suspended in glycerol were cultured in Petri dishes with $YEB_{KAN50}$ medium [81] and grown for 2 days at a temperature of 28 °C.
(b) The bacteria were cultured on minimal medium $AB_{KAN50}$ [82] and grown for 1–2 days at 28 °C.
(c) A single colony was inoculated into 10 ml of liquid $YEB_{KAN50}$ medium in 100 ml Erlenmeyer flasks and grown on vortex mixer at 250 rpm at 28 °C for 16–20 h.
(d) Fresh mg/L $_{KAN50}$ [83] medium was inoculated with *A. tumefaciens* suspension at a ratio of 1:400, and culture was continued at 28 °C to obtain late log phase of bacterial growth with an $OD_{550}$ of 1.
(e) The culture was vortexed for 10 min at 10,000 rpm at 4 °C. Supernatant was discarded, and the bacteria in the deposit were resuspended in a fresh $MSGA_{KAN50}$ medium (based on Murashige and Skoog medium [84] with 1 μM of acetosyringone and 1 M of glucose). The ready suspension was poured onto Petri dishes which were used for inoculation.

A range of different standard media was used for culture of *A. tumefaciens* containing pBI-CRSA gene construct. The prepared media were sterilized in an autoclave for 20 min at 121 °C. The filtered selective antibiotic kanamycin was added to the sterile media in a concentration of 50 mg/L.

### 3.3 The Transgenesis of Sage Apical and Axillary Buds Was Conducted in Two Stages

Firstly, the initial transformation was carried out on 120 apical and axillary bud explants derived from 3-week- and 2-week-old multiplants and seedlings. Explants

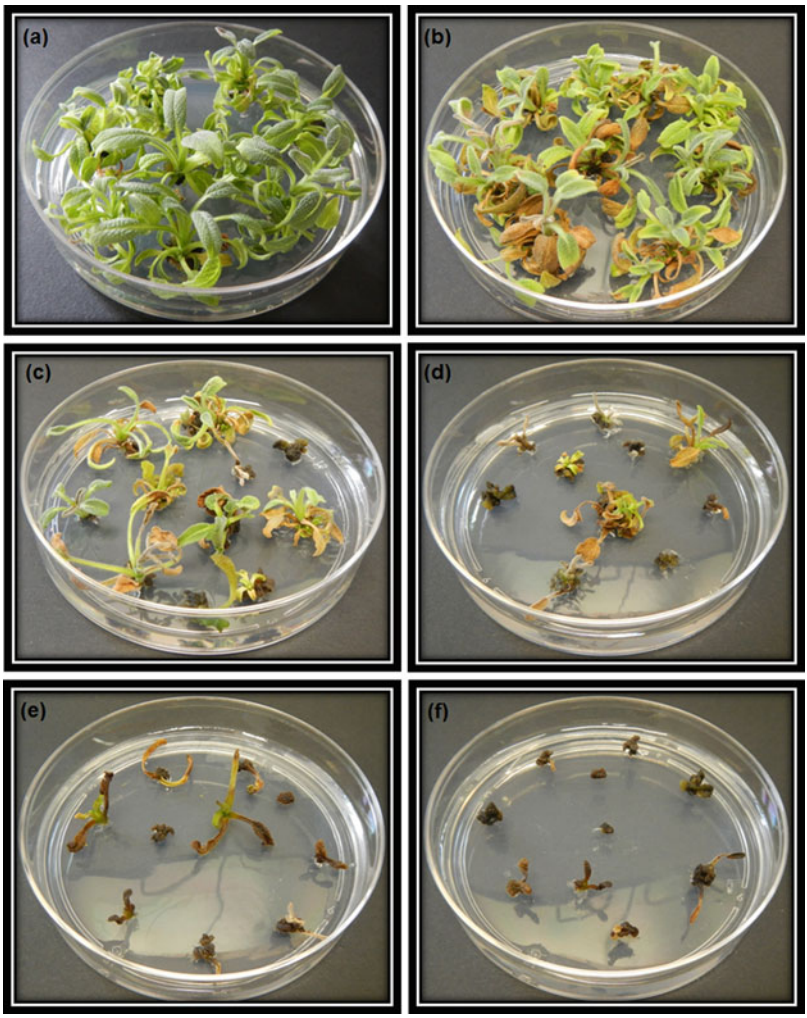


**Fig. 3** Establishing lethal kanamycin concentration for sage multiplant explants. (**a**) Positive control without kanamycin, (**b**) 25 mg/L, (**c**) 50 mg/L, (**d**) 100 mg/L, (**e**) 150 mg/L, (**f**) 200 mg/L lethal concentration

were described in detail in order to establish the influence of the explants' shape and size on its susceptibility to transformation. Secondly, on the basis of the results obtained in the first stage, 400 explants from apical and axillary buds derived from 3-week-old multiplants were selected for transformation.

At each stage a negative control (non-transformed explants on a selection medium) and a positive control (non-transformed explants on a medium without a selecting agent) were prepared for each kind of explants.

The explants that underwent transformation were placed in *A. tumefaciens* cell suspension for 10–15 min. Following the inoculation period, the buds were blotted dry on sterile tissue paper and placed on MS medium with 0.3 mg/L BA. A coculture was then grown for 2–3 days at 28 °C in semi-shade. In the next stage, excessive bacteria were removed by rinsing the explants in sterile water. The explants were then blotted dry on tissue paper and placed on $MSKAN_{200}$:$T_{250}$ selection medium with BA phytohormone and kanamycin and Timentin®. In both stages of the experiment, the selective culture was monitored in 2-week intervals before subsequent passaging. The observations involved assessing the state of the explants (growing, vitrificated, necrosed). With longer culture time, multiplication rate of the plants and the presence of *A. tumefaciens* were determined. *A. tumefaciens* did not cause infection in the subsequent subcultures, which enabled for using a medium without Timentin®. Moreover, in the second stage of the experiment, after the eighth passage, it was decided to grow the culture without a selecting agent. In both stages of the experiment, the rooting of explants growing on the selection medium was tested. In the first stage, rooting was conducted on one-half MS medium containing 0.3 mg/L IAA with a selecting agent, while in the second stage, the rooting medium did not contain any selecting agent.

## 3.4 The Transgenesis of Sage Meristems of Apical and Axillary Buds

In the next stage of the research, isolated apical and axillary sage meristems were also subjected to transformation. Explants of less than 1 mm in size consisted almost exclusively of meristematic tissue so that bacteria would reach meristematic cells more easily, thus increasing the probability of their transformation. Transformation of isolated meristems was performed by directly injecting a needle inserted in *A. tumefaciens* suspension into the meristems under a binocular microscope. Thus prepared explants were placed on MS medium with 0.3 mg/L BA. A coculture was then grown for 2–3 days at 28 °C in semi-shade. In the next stage, excessive bacteria were removed by rinsing the explants in sterile water. The explants were then blotted dry on tissue paper and placed on two kinds of mediums: $MSKAN_{200}$:$T_{250}$ regeneration selection medium (0.3 mg/L BA) containing Timentin® and kanamycin (200 mg/L) and regeneration medium MST250 (0.3 mg/L BA) containing only Timentin® in the concentration of 250 mg/L. Due to the small size of the isolated meristems, regeneration medium without a selecting agent was used. Selecting agent was introduced when explants began to expand into shoots. Meristems were derived both from seedlings and 3-week-old multiplants. Meristems obtained from seedlings (40 explants each) were inoculated with *A. tumefaciens* in the concentration of $10^8$ and $<10^8$, additionally in an injected/non-injected combination (160 explants). There were three controls performed: two positive (MS 0.3 mg/L BA and MST250 0.3 mg/L BA) and one negative ($MSK_{200}$:$T_{250}$ 0.3 mg/L BA). Each control consisted of 20 explants, non-injected and injected with the preparation needle (120 explants). The first examination of the explants was

performed 2 weeks after rinsing the coculture explants and then after another 4 weeks since the first observation. After 5 rounds of selection explants subcultures, antibiotics were no longer administered.

Three explants growing on a selection medium were selected after the transformation. DNA was isolated from these samples following modified Gawal and Jarret (1991) method [85], and then transgene integration analysis was conducted using PCR. However, despite the fact that the plants grew on selection media, our research did not confirm the presence of transgene in the plant cells. It was suspected that temporary transfection took place, and the introduced DNA did not integrate with the sage genome, producing only temporary resistance to the selective antibiotic. Thus, the introduced gene might have been lost during cell division in plant multiplication. This may explain the gradual decrease in the vitality of the explants in selection cultures until their complete decay. Explants were selected during the 3rd–5th subculturing following the transformation. Further passages, due to the small size of the plants, provided material both for DNA isolation (6th–11th subcultures) and assessment of the rhizogenesis process, which indicates normal development of a plant.

The lack of implemented fragments of the *S. mutans* gene in the genome of the explants analyzed might have been caused by the instability of transformant strains or loss of the transgene during passages. A stable transgenic line is produced only when the transgene integrates with the genome in the right location. Loss of the transgene in subsequent passages may be also caused by the activation of processes leading to the identification and repair of DNA changes in meristematic cells. Explant rhizogenesis after transformation was not consistent with the described sage regeneration methodology, proving that genetic homeostasis was disturbed and explants did not develop normally.

The type of explant selected determined the process of transformation; apical and axillary buds ensured quick and effective regeneration. However, as the explants contained both meristems and leaf primordia, it was difficult for *A. tumefaciens* to reach the meristematic tissue. After a detailed analysis of all explants, transformation of isolated meristems was performed by directly injecting a needle inserted in *A. tumefaciens* suspension into the meristems. The aim was to ensure that the bacteria could reach the meristematic cells more easily and increase the probability of their transformation. Although several variants of the experiment were conducted, the stable transgene integration in producing plants was not obtained. However, valuable observations were made in control explant cultures regarding high development potential of isolated meristems. This type of culture may be used in the future to produce transgenic plants using nonvector methods.

Secondary metabolite content was measured in the raw material obtained in subsequent transformations of sage (Agro 1, Agro 2, Agro 3) and compared with the material grown in in vitro cultures (Table 5).

Biochemical analysis showed no differences in the content of flavonoids, polyphenolic compounds, polyphenol acids, and tannins in the material obtained after the transformation and that from in vitro cultures.

**Table 5** Secondary metabolite content in sage leaves

| | Content [%] | | | | | |
|---|---|---|---|---|---|---|
| | Flavonoids per | | Tannins per pyrogallol | Polyphenolic compounds per rosmarinic acid | Polyphenol acids per chlorogenic acid | Loss of weight after drying |
| | Perquercetin | Perhyperoside | | | | |
| In vitro | 0.37 | 0.53 | 1.45 | 6.37 | 7.38 | 6.46 |
| Agro 1 | 0.38 | 0.54 | 1.38 | 6.38 | 8.10 | 5.30 |
| Agro 2 | 0.27 | 0.39 | 1.33 | 6.75 | 7.21 | 5.94 |
| Agro 3 | 0.24 | 0.35 | 1.52 | 5.97 | 6.89 | 6.33 |

Yet it is worth noting that the material obtained after the first transformation (Agro 1) contained the highest amount of quercetin, hyperoside, and chlorogenic acid compared to the raw material after the second and third transformations and a similar amount compared to the material from in vitro cultures. As regards the raw material after the second (Agro 2) and third (Agro 3) transformations, it contained the highest amount of rosmarinic acid and tannins, respectively.

Transformation of explants without producing transformed plants did not affect the content of the sage active compounds analyzed as compared to the material derived from in vitro cultures.

The main goal of research studies whose aim is to improve medicinal plants by the use of biotechnological methods is to increase the synthesis of secondary metabolites [86]. However, in the presented research with the use of *A. tumefaciens*, the stable integration of the *Streptococcus mutans* gene fragments in sage genome was not achieved. Paradoxically, in the case of sage, the biosynthesis of active ingredients may inhibit *A. tumefaciens* growth and thus become an obstacle for producing genetically modified plants by the use of vector-based transgenesis systems. For that reason the influence of sage on the growth and development of *A. tumefaciens* colonies was examined.

## 4 The Influence of Sage on *Agrobacterium tumefaciens*

Sage displays high antibacterial activity against numerous microorganisms. It has been demonstrated that the compounds found in sage essential oil inhibit the multiplication of pathogenic bacteria, both gram-positive, i.e., *Staphylococcus aureus* and *Bacillus subtilis*, and gram-negative, i.e., *Escherichia coli* and *Pseudomonas aeruginosa* [9]. Moreover, they have a virucidal effect. Stanojevic et al. described the synergistic activity of antibacterial sage aqueous extracts and preservatives against selected bacteria, including *A. tumefaciens* [22]. Their research showed that the minimum inhibitory concentration (MIC) for the growth of *A. tumefaciens* was 20 mg/mL, which resulted in medium antibacterial activity compared with *B. subtilis* (MIC 10 mg/mL) and *E. coli* (MIC 40 mg/mL), but did not guarantee bacterial resistance. The research showed synergistic action of sage extracts with preservatives; sage extract in a concentration of 1 mg/mL with sodium nitrite effectively inhibited the growth of *A. tumefaciens*. For this reason, in the presented experiment, the influence of sage explants placed in synthetic media on bacterial growth during inoculation and coculture was determined. The experiment was performed on MS medium with 0.3 mg/L BA (6-benzylaminopurine) in three variants (Fig. 4):

1. Experimental coculture of *A. tumefaciens* with sage explants, which involved culturing the bacteria in a straight line and placing sage explants next to it on one side. Apical buds from 3-week-old multiplants were used as sage explants, as was done during the transformation. Seedlings and leaves with petioles were used as

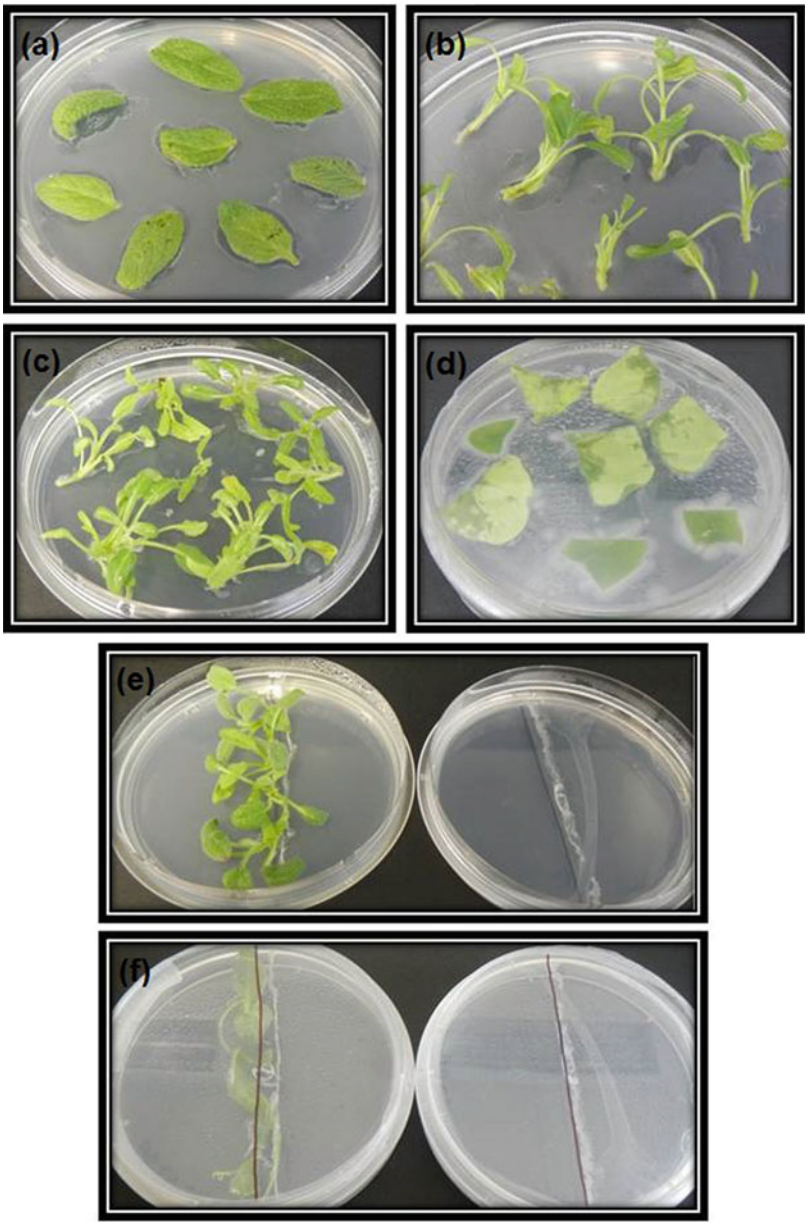


**Fig. 4** The effect of sage explants on *A. tumefaciens* growth on the third day of coculture. (**a**) The inhibition of bacterial growth around sage leaves (second variant); (**b**) the inhibition of bacterial growth around apical buds (second variant); (**c**) the inhibition of bacterial growth on apical sage buds previously placed in *Agrobacterium* suspension (third variant) visible lack of bacterial growth on sage explants; (**d**) bacterial growth on tobacco explants previously placed in *Agrobacterium* suspension; (**e**) growth inhibition of bacteria inoculated linearly beside explants from sage seedlings as compared to the control (first variant); (**f**) bacterial growth inhibition in the presence of sage leaves as compared to the control (first variant)

well. The experiment was conducted in three replicates with 8 explants of each type per replicate.
2. Bacterial coculture cultured on the entire surface of the dish, with apical buds or leaves placed on it. The experiment was conducted in two replicates with 8 explants of each type per replicate.
3. Coculture in which apical buds were soaked in bacterial suspension for 15 min in order to establish the scale of *A. tumefaciens* growth on explants. In addition, a coculture of tobacco leaf fragments soaked in bacterial suspension was established in order to compare the influence of the two plant species on the growth and development of *A. tumefaciens*.

LBA4404 *A. tumefaciens* strain inoculated into the medium was in the same log growth phase (OD ~ 1) as the bacteria used in the transformation. The same strain of bacteria inoculated without sage explants was used as a control. Cultures were grown in semi-shade at 26 °C. The growth and development of the bacteria and explants were examined four times in the coculture of sage explant with *A. tumefaciens*: after the first, third, seventh, and fourteenth days of the experiment. Observations following the first and third days after the inoculation were particularly important because that was the time of inoculation in the previously conducted transformation. In the first variant, following the first day of the coculture, both the bacteria cultured in a straight line and the sage explants placed next to them developed normally. In the second variant, inhibition in the growth and development of bacteria placed beside the leaves and a 75% growth inhibition of sage apical buds was observed. In the variant with sage apical buds, which had been placed in *A. tumefaciens* suspension, a limited bacterial growth was observed.

The second coculture examination conducted on the third day of the culture followed the protocol of sage transformation, which had been carried out using *A. tumefaciens* in the transgenesis process (Fig. 4). Both in the first and second variants, bacterial growth inhibition was observed with various intensity compared to the control. In the first variant, apical buds, seedlings, and leaves limited the growth of the linearly inoculated bacteria (Fig. 4e–f). Bacterial growth inhibition around the leaves and complete lack of bacteria under explants were observed for the bacteria inoculated in the entire medium (Fig. 4a). Apical buds inhibited bacterial growth mainly in those places where the explant was in contact with the medium (Fig. 4b). In the third variant, there was a slight growth of bacteria, with no growth on explants (Fig. 4c). All the sage explants grew normally with no stress symptoms. The state of the coculture did not change on the seventh day of the culture (third examination). After 14 days, the sage explants gradually turned brown and decayed due to the shortage of nutrients in the medium. At the same time, an increased growth of *A. tumefaciens* was observed.

The conducted analysis leads to the conclusion that sage explants had an inhibiting effect on bacterial growth and development. This might have decreased the probability of a successful introduction of a fragment of bacterial plasmid (T-DNA) into the plant genome. The similar observation was given by Marchev et al. [87], who noticed difficulties during *A. tumefaciens* transformation of

*S. tomentosa* plants due to the extensive release of phenolic compounds that had strong antimicrobial and allelopathic activities [87]. This negative effect was postponed by using a temporary immersion system in combination with a two-phase cultivation protocol that incorporates Amberlite XAD-4 resin. This procedure enabled to obtain 100% transformation efficiency using *S. tomentosa* mature leaves.

## 5 Conclusions

Progress in plant biotechnology can also be observed in the context of medicinal plant species. Development of effective regeneration methods for plants which are crucial in phytotherapy, such as sage, creates a multitude of possibilities for their better use: exploring metabolic pathways and increasing production of secondary metabolites, micropropagation of uniform genotypes and their long-term storage, genetic transformation, and the production of biopharmaceuticals. The present research points to considerable difficulties in sage transgenesis using vector-based methods with *A. tumefaciens*. The problem of achieving stable transgene integration in sage is related to its high antibacterial activity against a wide range of microorganisms. The observed negative effect of active ingredients contained in sage explants on bacterial growth and development is a major obstacle for conducting an effective sage transformation using *A. tumefaciens.* Nonvector methods should be recommended to obtain the successful transformation of sage due to its strong antibacterial effectiveness.

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