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3 **Isoflavone production in hairy root cultures and plantlets of *Trifolium pratense***

4

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22

1 **Abstract**

2

3 *Objectives*

4 The aim of this study was to develop a *T. pratense* hairy root (HR) production protocol and
5 select HR lines with high isoflavone yield following elicitor treatments.

6 *Results*

7 We obtained 13 independent HR lines, producing approximately 3 times more isoflavonoids
8 than seedlings (3.3 mg/g dry weight) and in which 27 isoflavonoids were detected. Each HR
9 line had its own isoflavonoid profile. These lines produced as major components daidzein,
10 genistein, formononetin and biochanin A. Sucrose, salicylic acid (SA), yeast extract (YE) and
11 flagellin 22 (flg22) were tested as elicitors. Using SA 140 mg/L, allowed the maximum
12 isoflavonoid production in plantlets (11.9 mg/g dry weight) but reduced root growth, possibly
13 as a result of its toxicity. The highest isoflavone production in HR (27.9 mg/g dry weight) was
14 obtained using sucrose 60 g /L, for 3.5 days.

15 *Conclusion*

16 This work reports the high production of various isoflavonoids with *T. pratense* elicited HR
17 cultures.

18

19 **Keywords:** Elicitors; plantlets; red clover; salicylic acid; sucrose.

20

21

1 Introduction

2 Red clover (*Trifolium pratense* L.) is a forage legume producing as main isoflavones
3 formononetin, biochanin A, daidzein and genistein (Fig.1-A to D) (Saviranta et al. 2008).
4 These molecules present several biological activities related to their phytoestrogenic-like
5 activity, due to some similarities with the structure of 17 β -estradiol (Fig. 1-e) (Monteiro et al.
6 2018).

7 **Insert Fig. 1**

8 These economically interesting plant secondary metabolites generally accumulate in
9 roots (Saviranta et al. 2010). To avoid destroying the plant, the *Agrobacterium rhizogenes*
10 transformed hairy root (HR) cultures represent a good alternative for biotechnological
11 applications (Guillon et al. 2006; Putalun et al. 2007).

12 HR cultures are cytologically stable, exhibit continued growth without any hormonal
13 supplement, have negative gravitropism and produce opines. This stability allows biochemical
14 and genetic manipulations (Webb et al. 1990) and the development of controlled cultures
15 (Spagnuolo et al. 2014). To date, two studies have used red clover HR (Beach and Gresshoff
16 1986; Webb et al. 1990), but none of them focused on the secondary metabolites production.

17 Plants produce various natural products as part of their development or in response to
18 elicitors that can be used to increase secondary metabolite production (Shinde et al. 2010;
19 Udomsuk et al. 2011). Yeast extracts (YE, *Saccharomyces cerevisiae*) and salicylic acid (SA)
20 can be used as elicitor in HR cultures (Ramirez-Estrada et al. 2016; Shinde et al. 2009b;
21 Udomsuk et al. 2011). The bacterial flagellin or part of it (flg22 peptide) is also a potent
22 elicitor, eliciting basal plant defenses in plants like *Arabidopsis* (Maffei et al. 2012; De
23 Coninck et al. 2015). Sucrose has important signaling functions throughout all stages of the
24 plant life cycle (Smeekens 2000; Sujatha and Kumari 2012). At high concentrations it can
25 trigger reactive oxygen species production and also polyphenol, including anthocyanin,

1 accumulation in grape (*Vitis vinifera*) (Ramakrishna and Ravishankar 2011) and also
2 flavonoids and anthocyanins production in *A. thaliana* (Solfanelli et al. 2006) .

3 The aim of our study was to develop a *T. pratense* hairy root (HR) production protocol
4 and select HR lines with high isoflavone yield following elicitor treatments. We report a
5 medium term (five months) study for the production of daidzein, genistein, formononetin and
6 biochanin A in HR culture.

7 **Experimental**

8 *Plant material*

9 Seeds of *T. pratense* var. URS-BRS Mesclador were donated by Dr. Miguel
10 Dall'Agnol (UFRGS). The specimen was deposited under the registration code HAS 87114,
11 number 4291 (10/14/1986) (Museum of Natural Sciences - Zoobotanic Foundation of Rio
12 Grande do Sul). The harvest point is (latitude: -30.0331/ longitude: -51.23 [err: ± 29946
13 WGS84]).

14 *Seed disinfection and germination*

15 In order to initiate the *in vitro* germination process, *T. pratense* seeds were scarified
16 with fine sandpaper and the disinfection process started with sodium hypochlorite (2% active
17 chlorine) plus tween 20 (two drops/ 100 mL) under stirring conditions for 30 minutes,
18 followed by triple washes with sterile water and inoculation in agar/ water medium (agar 8 g
19 /L). The seeds were maintained for 48 hours at 4 °C before their transfer to 24 °C (2 days) in
20 the dark. The seedlings have started to grow at this point (± 1.5 cm in length) and were shifted
21 to a growth chamber with 16 h photoperiod (200 µE/s/m²) in ½ MS medium (Murashige and
22 Skoog 1962) plus sucrose 10 g /L and agar 7 g /L (Supplementary material) .

23 *Screening for elicitor effects on the roots of T. pratense plantlets*

1 In order to test the elicitor effects on *T. pratense* development, the experiment was
2 initiated with 5 days-old *T. pratense* seedlings that were cultivated in the same conditions as
3 described above. The elicitation effect was measured at 0, 12, 24, 36, 48 and 72, 96, 120 or
4 168 h after inoculation.

5 *Elicitor preparation and treatment*

6 Elicitors treatment was tested using the Flagellin 22 peptide
7 QRLSTGSRINSAKDDAAGLQIA (flg22, Proteogenix, Schiltigheim France), yeast extract
8 (BD Biosciences), salicylic acid (Sigma Aldrich) and sucrose. The elicitor action was
9 estimated as the percentage of root growth inhibition after treatment. Seedlings were grown
10 on agar 7 g /L or PhytigelTM 2 g /L as gelling agents. Elicitors were dissolved in distilled
11 sterile water and added to the medium in the following concentrations. Flg22 was used in the
12 concentrations of 0; 0.25; 0.5; 1 and 2.5 μ M (Millet et al. 2010). The YE (Supplementary
13 material) was tested at 0.5; 1; 2; 3 and 4 g /L of medium. YE solution was filter sterilized and
14 added after the medium sterilization (Sivesind and Seguin 2006). SA was dissolved in
15 distilled water and autoclaved at 121 °C for 15 min. After sterilization, SA was added at
16 concentrations of 0, 1.4, 14, 70 and 140 mg /L. Sucrose (Sigma Aldrich) was employed from
17 10, 30, 60, 90 until 180 g /L and was autoclaved with the medium.

18 *Hairy root culture*

19 a) *Agrobacterium* culture and tissue infection

20 The *A. rhizogenes* strain A4TC24 (Petit et al. 1983) was cultured in the flask
21 containing 4 ml of YEB liquid medium + rifampicin 100 μ g /mL. The incubation was done at
22 30°C, in a rotary shaker at 200 rpm. One day before the transformation, 100 μ L of the liquid
23 culture was plated on a YEB agar plate with appropriated antibiotics. The culture was

1 incubated overnight at 30°C to obtain a layer of *A. rhizogenes* on the plate to proceed with the
2 transformation.

3 The 7 days-old seedlings were infected aseptically by cutting the root meristem and
4 dipping it into the *Agrobacterium* layer or by wounding the principal root using
5 *Agrobacterium*-filled syringes. Inoculated seedlings were kept for two days in ½ MS, storing
6 the plate with an angle of 45° and the root covered with opaque black plastic film. After this
7 time, the infected seedlings were transferred to ½ MS + 400 µg /mL Amoxicillin Na and
8 clavulanate K 5:1 + 1% sucrose for 15-21 days (photon flux of 40 µmol m⁻² s⁻¹), 25 ± 2 °C,
9 with the roots covered to remain in the dark (Wright and Wang 2015).

10 When the new growing HR reached the size of 10-15 cm, they were cut from the plant
11 and transferred to new medium, with the same components and sub cultured each thirty days.
12 Roots growing efficiently after two subcultures independently of the plant were considered as
13 true HR. These HR were transferred to Erlenmeyer flasks containing 50 mL of ½ MS liquid
14 medium and maintained at 20 °C on a shaker (100 rpm) in the dark. These were also sub
15 cultured every 30 days.

16 *Evaluation of the isoflavone content in seedlings and HR cultures*

17 The seedlings were grown for seven days using the media including the elicitors (sucrose +
18 phytigel, sucrose + agar, SA, YE and Flg22), as cited above (*Elicitor preparation and*
19 *treatment* section) and harvested for analysis. Thirteen HR lines with good growth capacities
20 were isolated and partly harvested each 30 days of culture. The material was stored at -80 °C
21 for posterior flavonoids extraction and control of isoflavone content during the first 5 months
22 of the experiment.

23 The flavonoids analysis by Ultra Performance in Liquid Chromatography (UPLC) was
24 adapted from the literature (Galland et al. 2014) and the samples were compared to standards

1 of the commercially acquired isoflavones daidzein, genistein, formononetin and biochanin A
2 (Sigma-Aldrich). The hairy root cultures were lyophilized, and the extraction made using
3 around 100 mg of *T. pratense* material reduced to powder using liquid nitrogen, mortar and
4 pestle. To this powder, 1.5 mL of extractive solution (methanol/ water/ acetone/ TFA, v/v 40/
5 32/ 28/ 0.05 %) was added and the membranes were further disrupted by placing the samples
6 in an ultrasonic bath for 20 min at 25 kHz at 4 °C. These samples were then centrifuged at 20
7 000 g for 20 min. The process of extraction was performed twice with the same sample and
8 the two resulting supernatants were pooled and dried in a rotary evaporator and freeze dryer.
9 The dry pellet was dissolved in acetonitrile (ACN): water (1:1 v/v) and filtered in 22 µm
10 (Millipore).

11 a) UPLC isoflavones quantitative analysis

12 The *T. pratense* HR culture and seedling extracts were analyzed by Acquity UPLC
13 (Waters) equipped with a Waters Photodiode Array Detector eλ UV detector. The separation
14 of the compounds was achieved in a reverse phase (Acquity UPLC BEH C18, 1.7 µm, 2.1 x
15 50 mm, Waters), using 3 µL of each sample in a flow rate of 0.3 µL /min, at 40 °C and a
16 binary gradient: (A) water and formic acid 0.1% (V/ V) and (B) ACN and formic acid 0.1%
17 (V/ V). The solvent gradient was programmed as follow: 0-2 min 5% B, 2-4 min 10% B, 4-17
18 min 40% B, 17-21 min 100% B, 21-23 min 100% B. The wavelength analyzed was at 260
19 nm.

20 Quantitative analyzes were performed using commercial isoflavone standards to verify
21 the UV absorption, retention time and the linearity. Each curve was made with six different
22 concentrations. For daidzein the concentration range used was 1.5 – 5.5 µg /mL, for genistein
23 4.3 – 216.4 µg /mL, for formononetin 76.3 – 225 µg /mL and for biochanin A 5.9 – 357.2 µg
24 /mL. The linear equation and the determination coefficient (R²) were calculated for each
25 isoflavone standard curve.

1 b) Isoflavone identification

2 The following flavonoid extraction protocol was adapted from the literature (Galland
3 et al. 2014). HR flavonoids were extracted from 100 mg of 13 different HR *T. pratense* lines
4 cultivated for 60 days, after grounding in liquid nitrogen using mortar and pestle. Then, 1 ml
5 of methanol 80% (v/v) was added to the HR powder samples and membranes were further
6 ruptured by placing the samples in an ultrasonic bath for 20 min at 25 kHz at 4 °C and later
7 on a rotating wheel for 1 hour.

8 After centrifugation during 10 min at 20000g at 4 °C, the pellet was extracted with 1
9 ml of methanol 80% (v/v) overnight at 4°C on a rotating wheel. The two resulting
10 supernatants were pooled, filtered at 0.45 µm (Millipore) and dried on a SpeedVac for 8 h.
11 The dry pellet was dissolved in methanol/ water/ acetone/ trifluoroacetic acid (40/32/28/0.05,
12 v/v). At this stage, 20 ng of rhamnetin (Extrasynthese) were added as internal standard.
13 Finally, the extract was filtered again. The extracts were then analyzed by HPLC-
14 electrospray-MSMS (HPLC-ESI-MS/MS).

15 The compounds were introduced in the ESI source using a Waters UPLC Acquity I-
16 Class (Waters) equipped with a Waters PDA detector. Separation was achieved on a reverse-
17 phase column (Uptisphere C18 ODB, 150*2.1 mm, Interchim) using a flow rate of 0.40 mL
18 /min and a binary gradient: (A) acetic acid 0.1% (v/v) and (B) acetic acid 0.1% (v/v) in
19 acetonitrile. The solvent gradient was programmed as following: 0–4 min 10% B, 4-17 min
20 60% B, 17–23 min 100% B, 23-25 min 14% B (Kerhoas et al. 2006).

21 Analyses were performed on a Waters Xevo TQ-S (Waters) MS triple quadrupole mass
22 spectrometer operating in MS full scan for the relative quantification and identification of the
23 compounds, ESI positive and negative modes and In Source-Collision Induced Dissociation
24 (IS-CID) positive modes. Relevant instrumental parameters were set as following: cone
25 voltage 20 V and 80 V for usual and IS-CID modes respectively, capillary 2.70 kV, source

1 block and desolvation gas temperatures 150°C and 600°C, respectively. Nitrogen gas was
2 used to assist nebulization and desolvation (7 bar and 1000 L/h, respectively). When MS/MS
3 modes were used for the confirmation of the identification (Product ion scanning and
4 Precursor ion scanning 20 eV was used as collision energy in the collision cell).

5 *Hairy root elicitation*

6 Elicitation was done on 5 months stabilized cultures. For this, approximately 7 days
7 after subculture, hairy root cultures were transferred to ½ MS medium containing: sucrose 10
8 g/L (control) or sucrose 60 g/L or sucrose 10 g/L plus SA 10 or 30 mg/L as elicitors. The first
9 measurements were done after 3 days (72 h) of culture and at 84, 96, 108 and 120 h (3.5, 4,
10 4.5 and 5 days, respectively). For control (sucrose 10 g/L), the measurements were done until
11 the 14th day in culture. The HR cultures were collected, lyophilized, grounded and extracted
12 for total isoflavonoids measurement. The extraction and chemical analysis of isoflavones was
13 performed according to the technique described in *UPLC isoflavones quantitative analysis*
14 section.

15 *Statistical analysis*

16 All culture experiments were conducted in triplicate. The root length experiments were
17 composed of different elicitor treatments at different concentrations. For each treatment 5
18 replicates of three plates were used, considering each plate as an experimental unit. HR
19 cultures were run in quadruplicates for each line and the replicates of each line were
20 combined in two, prior to isoflavones extraction and analysis. Elicitation of the HR cultures
21 was performed in triplicates. Isoflavone concentrations were determined by means of the
22 standard curves for each one of the isoflavones analyzed in the samples, as well as the
23 standard deviation of the samples under study and Analysis of Variance (ANOVA) and Tukey
24 test at 5% significance level (Machado and Conceição 2002).

1 **Results and discussion**

2 *Effect of elicitor on roots length and isoflavone content in T. pratense plantlets*

3 In order to estimate the effects of different elicitors on *T. pratense*, we tested their
4 influence on seedling root growth and isoflavones content. Seedling root growth inhibition is
5 easier to test than HR growth inhibition, because it is fast and easy to measure on many
6 conditions. Compared to the control condition (sucrose 10 g/L) the different treatments either
7 stimulated or inhibited root growth. We postulated that the elicitor action will mimic defense
8 reactions and will inhibit root growth in these experiments. This was observed for high
9 sucrose (90 and 120 g/L) and SA (140 mg/L) treatments. Root growth stimulation rather
10 reflect a positive nutrition effect and was obtained in the treatments using YE 4 mg/L (106.4
11 mm), which did not differ statistically from SA 1.4 mg/L (105.6 mm), YE 1 mg/L (94 mm)
12 and sucrose 60 g/L + agar (88.9 mm) (Table S1–supplementary material).

13 These experiments were done using agar and phytigel as gelling agents. Roots were
14 growing slightly better on phytigel with sucrose at 10 g/L (Fig. 2-A), and similarly at 30 and
15 60 g/L. At sucrose concentration of 90 and 120 g/L the root growth inhibition was less
16 pronounced using agar as compared to phytigel.

17 **Insert Fig. 2**

18 Similarly, in *Arabidopsis thaliana* seedlings, sugar promoted growth at low
19 concentrations, although, at high concentrations, it acts synergistically to abscisic acid to
20 inhibit growth (León and Sheen 2003). Agar is routinely used as a gelling agent for tissue
21 culture; however, it is known that depending on the sources from which it comes, it may
22 contain impurities interfering with the experiments (Cosson et al., 2006). PhytigelTM, a
23 selected grade of gelrite, is a very clean gelling agent, very effective in different applications
24 (Veramendi et al. 1997). Despite that gelling agents should be inert constituents of the culture

1 media (Arregui et al. 2003), in our red clover seedlings root length experiment phytigel and
2 agar were similar at low sugar concentration (until 60 g/L) but plants grew better on agar
3 (Figure 3-A) at higher sugar concentration.

4 **Insert Fig. 3**

5 This experiment (Fig. 3 –A, Table S1) shows that total *T. pratense* isoflavones content
6 could be enhanced at high concentrations of sucrose in combination with agar, with a
7 production reaching 11.3 mg/g dry weight using 120 g/L of sucrose when it is only 3.2 mg/g
8 dry weight using 10g/L sucrose. Using phytigel the best isoflavones production (6 mg/g dry
9 weight) was obtained with 90 g/L of sucrose. Although the high sucrose and agar
10 concentrations were the most effective, they were extremely damaging to the seedlings root
11 growth (Fig. 2–A). Plantlets developed on phytigel showed a colour change at 90 g/L of
12 sucrose, with the formation of magenta colour near the cotyledons which correlated with the
13 colour of the flavonoid extracts obtained from them (Fig. S1). Thus, our study also indicates
14 that in *T. pratense* seedlings sucrose induces phenylpropanoid production with both an
15 increase in anthocyanin and isoflavonoid production (Fig. 3-A and Fig. S1).

16 SA at low concentration (1.4 mg /mL; Fig. 2–B) increases root growth. At 14 mg /mL
17 there is no change to control and at higher concentrations, root growth is inhibited. SA
18 treatment did not change isoflavonoid production until the concentration reaches 140 mg/L
19 (Fig. 3–B) by which the flavonoid amount almost tripled (11.9 mg/g dry weight). Similar
20 results were described with cell cultures and hairy root cultures of *P. corylifolia* (Shinde et al.
21 2009a) and for *Phaseolus vulgaris* cotyledons (Durango et al. 2013).

22 Contrary to sucrose and SA, YE (Fig. 2–C) had a rather positive effect on *T. pratense*
23 seedling root growth. The isoflavonoids production in these YE treated roots decreased for
24 0.5 and 1 mg/L (Fig. 3-C), was similar to the control for 2 mg/L and increased slightly above
25 the control for 4 mg/L YE. In studies with *P. candollei* HR cultures, YE was the most

1 efficient elicitor for flavonoid production (Udomsuk et al. 2011) and in *P. corylifolia* cell
2 cultures and HR, YE was the best elicitor, inducing higher levels of daidzein and genistein in
3 both studies (Shinde et al. 2009a; Shinde et al. 2009b). Thus, in contrast to previous studies,
4 at the concentration used YE did not behave as an elicitor but rather promoted growth and
5 was not used further in our experiments.

6 Flagellin 22 (flg22) is a conserved 22-amino acid-long N-terminal part
7 (QRLSTGSRINSAKDDAAGLQIA) of the main bacterial flagellum protein that can act as a
8 potent biotic elicitor in plants. For *T. pratense* seedlings, the different concentrations tested
9 did not change root growth significantly (Fig. 2–D) but increased slightly isoflavonoids
10 production, using 0.5 $\mu\text{M/L}$ (Fig. 3–D). As for YE, flagellin was not used further in our
11 experiment due to its poor elicitor effect on *T. pratense*.

12 All together isoflavonoid production was inversely related to the root growth
13 inhibition (elicitor effect), validating the elicitor testing protocol, and the best effects were
14 obtained with SA and sucrose that multiplied isoflavonoid production by three.

15 *Establishment of the Hairy Root (HR) cultures*

16 Hairy roots are induced by the transfer to the plant recipient genome of the *A.*
17 *rhizogenes* T-DNA sequence containing plant oncogenes (Petit et al. 1983). These oncogenes
18 allow formation of root cultures that also produce opines. The transformed nature of the roots
19 is attested by the high HR growth rate in absence of hormones, their negative gravitropism
20 and the constancy in metabolite yield due to their genetic stability (Chandra and Chandra
21 2011).

22 *T. pratense* seedlings were infected with the *A. rhizogenes* strain A4TC24 five days
23 after germination. They showed visible adventitious root development 10 days after
24 inoculation for about 90% of them, with root formation at the site of infection (Fig. 4–A). The

1 putatively transformed roots were cut and transferred to solid medium (Fig. 4–B) where they
2 showed rapid growth and loss of the response to gravity (negative gravitropism; Fig. 4–C).
3 Rapid elongation is a phenotypic characteristic of such transformed roots, and the rapid lateral
4 branching is responsible for the increase in mass and accumulation of these roots (Eskandari-
5 Samet et al. 2012). When properly developed, the cultures were shifted to liquid culture
6 medium (Fig. 4–D) where the multiplication rate was exponential (Fig. 4–E to H). All these
7 characteristics demonstrate that the root cultures obtained are true HR. Using this protocol,
8 thirteen *T. pratense* HR cultures were established and further studied.

9 **Insert Fig. 4**

10 a) Isoflavone identification

11 The thirteen HR cultures were harvested after the second month of culture to analyze
12 their total isoflavonoids content by U-HPLC-ESI-MS/MS. According to these analyses 27
13 compounds were found in the chromatograms of the extracts obtained from these cultures
14 (Table 1, compounds **1** to **27**). Among these compounds it was possible to identify both
15 aglycones and conjugated forms such as glycoside and glucoside malonates (Table 1 and Fig.
16 5), in agreement with studies done using *Trifolium* roots (Saviranta et al. 2010).

17 **Insert Fig. 5**

18 Fig. 5 shows that all the cultures were isoflavone producers, with the biochanin A (**4**)
19 isomers as major compound representing 26.3 to 58.0 % of the total isoflavonoid content. The
20 second most abundant molecule was the formononetin aglycone (**14**), representing 10.1 to
21 36.5 % of the compounds, followed by other isoflavonoids, such as irilone/ afrormosine-
22 malonyl hexose (**9**; 4.4 to 17.7 %) and biochanin isomers (**6**; 6.7 to 13.5%). Other molecules
23 were produced in minor quantities and some of the cultures were unique in having certain
24 molecules. For example, the HR10 culture was the only one to produce compounds **12** and **3**,
25 and the HR5 culture the only one producing compound **16**.

1 The HR cultures could be differentiated by their growth capacity and morphology, but
2 mainly by their biochemical production, demonstrating a high variability in isoflavonoids
3 types and content. These dissimilarities can result from genetic differences between the
4 seedlings used to start the HR cultures. Indeed *T. pratense* is an allogamous diploid plant and
5 individuals are genetically different.

6 Another important source of variation can be related to the effect of the *A. rhizogenes*
7 *rol* genes that can have different levels of expression between the different HR lines. This can
8 result in different auxins and cytokinins contents (or sensitivity) in each line (Fu et al. 2005).
9 These variations can produce differences in the accumulation patterns of secondary
10 metabolites as well as variations in growth rate and productivity between the clones. Previous
11 studies also showed that, even if the cultures come from a single root, they may present a
12 certain heterogeneity (Hu and Du 2006).

13 b) Isoflavone content in the first five months of culture

14 In order to study the stability of the red clover root cultures isoflavone profiles during
15 the first months after the transformation, four HR cultures (1HR, 2HR, 4HR and 6HR) were
16 selected based on their good multiplication rate and stability. At the time of liquid culture
17 subculture (each four weeks) part of the cultures were harvested and stored to analyze their
18 isoflavonoids content. Lines 1HR, 2HR, 4HR and 6HR were then evaluated for their daidzein,
19 genistein, formononetin and biochanin A content (Fig. 6–A to D and Table S2–Supplementary
20 material). Fig. 6–E shows the variations in the total isoflavone content of these molecules
21 over time for these four HR cultures.

22 Insert Fig. 6

23 The most abundant isoflavones described in red clover are biochanin A (**25**) and
24 formononetin (**22**), with the second being more present in roots (Saviranta et al. 2008). In
25 contrast, the transformed root cultures analyzed here presented different most abundant

1 metabolites. Line 4HR was the richest in daidzein (**27**, 1.73 mg /g) at the end of the 150 days
2 (Fig. 6–A, Table S2). Genistein was produced in large quantities in line 2HR (1.01 mg /g) at
3 120 days of culture (Fig. 6–B, Table S2) and formononetin (**22**) in line 6HR (20.79 mg /g)
4 after 3 months of culture (Fig. 6–C, Table S2). Biochanin A (**25**) was the main compound
5 detected in line 4HR (13.90 mg /g) after 4 months in liquid medium (Fig. 6–D, Table S2).

6 The total isoflavones content was also estimated in these four lines. At the first point
7 measured (30 days, Table S2) the total isoflavonoid content of the four HR cultures was three
8 time higher than the seedling one (9.4 to 11.6 mg/g dry roots versus 3.2 mg/g dry plant, Table
9 S1, sucrose 10 g/l and Table S2). The isoflavone content even reached 27 mg per g of dry root
10 in some cultures. Isoflavone production was stable for 1HR and 2HR over the 5 months of the
11 experiment. For 6HR an increase production was observed at month 3 and for line 4HR
12 production increased months 4 and 5 (Fig. 6–E and Table S2). Previous studies did not
13 address isoflavone production over several months. However, they have shown that HR lines
14 can maintain genotypes and stability for extended periods, only losing this ability with
15 drastic changes of the media or the addition of plant hormones that induce metabolic
16 modifications and cause disorganization in the cultures, as with *Datura* and *Nicotiana*
17 cultures (Doran 1997; Anil Kumar et al. 2018).

18 c) Elicitation of the isoflavonoid production

19 After the characterization, two lines, 4HR and 8HR, were selected to conduct
20 elicitation tests with sucrose 10 (control) and 60 g/L, as well as with salicylic acid 10 and 30
21 mg/L. After the treatment, the samples were harvested, stored and extracted for their
22 evaluation in daidzein, genistein, formononetin, biochanin A and the total content of these
23 four molecules using UPLC for a period of 3 to 14 days, depending on the sample analyzed.

24 In line 4HR grown in presence of sucrose 10 g/L (Fig. 7–A) the isoflavone total
25 concentration decreased during the culture from 11.21 mg /g at 3 days, down to 2.53 mg /g of

1 dry mass in the end of 14 days in culture. The highest production for daidzein (**27**),
2 formononetin (**22**), biochanin A (**25**) and total isoflavones was observed 3 days after
3 treatment (Fig. 7–A) and decreased slowly during the experiment. In the case of genistein it
4 was 8 days after treatment.

5 **Insert Fig. 7**

6 For the line 8HR (Fig. 7–B) the total isoflavones content at 3 days (9.77 mg /g)
7 increased slightly at 4 days and decreased to 6.81 mg /g after 8 days. The time determined as
8 best for the highest production was 4 days. The most abundant molecule for this control
9 culture was biochanin A (**25**). The isoflavone production decreased after 4 days.

10 Based on these results, isoflavonoid production following elicitation was only studied
11 for 4.5 days.

12 The use of 60 g/L sucrose in line 4HR (Fig. 7–C) increased the total isoflavones
13 production after 3.5 days of treatment to 27.88 mg /g. This is 2.5-fold the highest
14 concentration (11.209 mg /g) measured for the control experiment and nearly 10 time more
15 than in seedlings. Formononetin (**22**) increased 4 times, from 4.05 mg /g in control to 17.01
16 mg /g but genistein production remained low. At 3.5 and 4 days of treatment daidzein (**27**)
17 was produced in large amounts but at 4.5 days formononetin (**22**) was again the main
18 compound.

19 Using sucrose at 60 g/L for line 8HR (Fig. 7–D) resulted in a total isoflavones
20 production peaking after 3.5 days of treatment to 21.21 mg /g. Similarly, to what was
21 observed in line 4HR, the isoflavonoid production was different to the one observed in the
22 control culture. Formononetin (**22**) instead of biochanin A (**25**) was the most abundant
23 compound (14.76 mg /g) after 3.5 days of culture.

24 Different results were described in *Pueraria phaseoloides* transformed roots, where 30
25 g/L sucrose promoted better accumulation of isoflavones after 16 days of treatment (Liang et

1 al. 2004). In *Hypericum perforatum* L. root cultures, high quantities of sucrose were shown to
2 stimulate the production of total phenols and flavonoids (Cui et al. 2010), similar to what was
3 found in *P. corylifolia* root cultures, for which high concentrations, like 50 g/L, prolonged the
4 isoflavonoid biosynthesis and induced the accumulation even after more than 30 days of
5 culture (Shinde et al. 2010).

6 *T. pratense* hairy root cultures were tested using two SA concentrations (10 and 30
7 mg/L). The 4HR line was relatively insensitive to the 10 mg/L treatment (SA 10 treatment,
8 Fig. 7–E) with the best isoflavone production at 3 days (11.64 mg /g) similar to the control
9 one. In contrast, using 30 mg/L (SA 30 treatment, Fig. 7 – G) the flavonoid content (5.24 mg
10 /g) at 3 days of culture was low as compared to control culture and declined close to zero at
11 the end of 4.5 days. In both cases the treatment did not change drastically the relative content
12 of the different molecules.

13 The 8HR line produced more isoflavonoid (17.17 mg /g at 3 days) after the 10 mg/L
14 treatment than the control (Fig. 7–F) but this production decreased rapidly to control level in
15 the next sampling time. In the 30 mg/L treatment the production was high (12.20 mg /g) at 3.5
16 days but reduced importantly later. Note that the isoflavonoids profiles were different from
17 the control experiment. In the SA 10 experiment the main difference to control in the
18 isoflavonoid composition was an increase in daidzein (**27**) content and the absence of
19 genistein. Formononetin (**22**, 4.67 mg /g) and biochanin A (**25**, 5.74 mg /g) were the most
20 abundant, at 3.5 days with SA 30 after elicitation. Their production decreased toward the end
21 of the experiment.

22 Our experiments thus show that the isoflavonoid production can be stimulated in the
23 *T. pratense* HR cultures by addition of sucrose 60 g/L (Table S3–Supplementary material),
24 mainly for the 4HR line, increasing daidzein (**27**), formononetin (**22**) and the total isoflavones
25 content well above the values obtained with the seedlings. Note that genistein production was

1 the highest using control sucrose condition (10 g/L). In contrast, SA treatment had rather
2 limited and short-term effects on isoflavonoid production, increasing only biochanin A (25)
3 production 3 days after the treatment. Higher SA concentration had rather negative effects.
4 This is in contrast to the *P. corylifolia* HR study for which isoflavonoid production was
5 independent of the SA concentration and of the length of the treatment (Udomsuk et al. 2011).
6 Similar results for the negative effect of the SA treatments were reported in another study
7 (Shinde et al. 2009b).

8 **Conclusion**

9 In this work, we have produced *A. rhizogenes*-mediated HR culture lines of *T.*
10 *pratense* and studied their isoflavone content. Both the total or main aglycone isoflavones
11 present in seedlings and HR cultures were determined and their production in the presence of
12 elicitors tested. Seedlings had better root growth in the presence of salicylic acid 1.4 mg/L
13 and yeast extract 4 mg/L, but higher elicitor concentrations inhibited growth. The highest
14 accumulation of total isoflavones in seedlings (11.9 mg/g dry weight) was found using SA
15 140 mg/L. The independent hairy root cultures showed fast growth rates during the five
16 months of the experiment and increased isoflavonoid production as compared to the seedling.
17 Interestingly these cultures exhibited different isoflavonoid profiles and these profiles
18 changed in the presence of elicitors, with high-level isoflavone production (27.9 mg/g dry
19 weight) when sucrose was added at 60 g/L in the two lines analyzed.

20 Thus, these experiments showed that the use of *A. rhizogenes* strain A4TC24 mediated
21 hairy root cultures in the presence of sucrose 60 g/L could be used to produce different *T.*
22 *pratense* isoflavonoids. The rather simple procedure described here can now be used as the
23 starting point to establish large-scale cultures in bioreactors using amplification of cultures as
24 described in Mehrotra et al., (2016) or for the manipulation of these cultures by expressing
25 specific genes allowing overproduction of specific molecules.

1

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13

14 **Compliance with ethical standards**

15 Conflict of Interest: The authors declare that they have no conflict of interest.

16 **Supplementary material**17 *Media*

18 All media were calculated for a volume of 1L and were sterilized by autoclaving for 20 min at
19 120 °C. Antibiotics, hormones or other components, were added after autoclaving the media.

20 1. Media for plant culture

21 MS medium (Murashige and Skoog 1962):

1 4.49 g Murashige and Skoog medium basal salt mixture including vitamins and
2 microelements (Duchefa.), 30 g sucrose pH 5.8, 1% agar (Difco,) for solid medium. Sterilized
3 by autoclaving.

4 2. Media for bacterial culture

5 a) LB medium:

6 5 g Yeast extract (Difco), 10 g Tryptone (Difco), 10 g NaCl, 10 g agar (Difco) for solid
7 medium, pH 7.0. Sterilized by autoclaving.

8 b) YEB medium:

9 5 g Beef extract (Difco), 1 g Yeast extract (Difco), 5 g Peptone (Difco), 5 g Sucrose, 2 mM
10 MgSO₄, pH 7.2. Sterilized by autoclaving.

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Tables Supplementary Material

Table S1. Root growth (mm) and isoflavone content (mg /g dry plants) on *T. pratense* seedlings grown in medium with elicitors at different concentrations during the first seven days after germination*.

<i>Elicitor</i>	<i>Dosage</i>	<i>Total isoflavone</i>	<i>Root lenght (mm)</i>	<i>Standard deviation</i>
<i>Sucrose + agar (g/L)</i>	10.0	3.20 D	67.9 EFG	0.163
	30.0	5.75 CD	84.8 CDE	0.227
	60.0	5.76 CD	88.9 ABCD	0.310
	90.0	3.91 CD	47.7 G	0.334
	120.0	11.34 AB	21.9 H	0.676
<i>Sucrose + phytagel (g/L)</i>	10.0	3.24 D	76.6 CDEF	0.440
	30.0	2.35 D	85.4 BCDE	0.088
	60.0	2.3 D	82.9 CDE	0.149
	90.0	6.26 BCD	20.7 H	0.205
	120.0	3.09 D	12.2 H	0.337
<i>Salicylic acid (mg/L)</i>	1.4	3.42 D	105.6 AB	0.144
	14.0	3.28 D	60.3 FG	0.191
	70.0	4.65 CD	16.7 H	0.095
	140.0	11.90 A	12.6 H	0.289
<i>Flagellin 22 (µM/ L)</i>	0.25	4.13 CD	83.8 CDE	0.089
	0.50	5.59 CD	69.3 DEF	0.112
	1.0	2.33 D	70.8 DEF	0.118
	2.0	4.26 CD	85.4 BCDE	0.203
<i>Yeast extract (mg/L)</i>	0.5	2.59 D	77.0 CDEF	0.088
	1.0	2.51 D	94.0 ABC	0.074
	2.0	3.35 D	82.9 CDE	0.151
	4.0	4.28 CD	106.4 A	0.137

*Results are the mean of three replicates \pm SD and ANOVA followed by Tukey test at 5% of probability. Different letters for the same treatment are significantly different at $P < 0.05$.

Table S2. Isoflavones and total isoflavones content of the *T. pratense* HR lines 1HR, 2HR, 4HR and 6HR during the first 5 months of culture.

<i>Analysis</i>	<i>Culture</i>	<i>30 days</i>	<i>60 days</i>	<i>90 days</i>	<i>120 days</i>	<i>150 days</i>
<i>Daidzein (mg/ g dry roots)</i>	1HR	0.48 ± 0.22 C	0.90 ± 0.11 B	0.09 ± 0.003 DE	0.00 E	0.21 ± 0.053 DE
	2HR	0.00 E	0.43 ± 0.02 C	0.00 E	0.00 E	0.71 ± 0.116 B
	4HR	0.43 ± 0.09 C	0.00 E	0.00 E	0.00 E	1.73 ± 0.01 A
	6HR	0.15 ± 0.06 DE	0.27 ± 0.01 CD	0.00 E	0.00 E	0.13 ± 0.007 DE
<i>Genistein (mg/ g dry roots)</i>	1HR	0.19 ± 0.07 EF	0.19 ± 0.05 EF	0.19 ± 0.06 EF	0.49 ± 0.06 C	0.24 ± 0.04 DE
	2HR	0.52 ± 0.04 B	0.00 G	0.00 G	1.01 ± 0.001 A	0.27 ± 0.03 DE
	4HR	0.13 ± 0.06 F	0.48 ± 0.03 C	0.48 ± 0.05 C	0.48 ± 0.05 C	0.00 G
	6HR	0.32 ± 0.04 D	0.23 ± 0.01 DEF	0.69 ± 0.01 B	0.17 ± 0.01 EF	0.00 G
<i>Formononetin (mg/ g dry roots)</i>	1HR	4.81 ± 0.90 EF	1.16 ± 0.24 JK	1.98 ± 0.62 IJ	3.44 ± 0.46 GH	3.04 ± 0.35 HI
	2HR	5.70 ± 0.48 DE	5.48 ± 0.18 E	2.68 ± 0.18 HI	5.64 ± 0.51 DE	5.35 ± 0.73 EF
	4HR	6.78 ± 0.06 D	4.27 ± 0.03 FG	2.18 ± 0.20 IJ	12.62 ± 0.60 C	13.87 ± 0.11 B
	6HR	5.63 ± 0.43 DE	5.12 ± 0.32 EF	20.79 ± 0.18 A	1.14 ± 0.08 JK	0.00 K
<i>Biochanin A (mg/ g dry roots)</i>	1HR	5.67 ± 1.14 B	1.47 ± 0.67 E	1.43 ± 0.71 EF	5.34 ± 0.61 B	2.97 ± 0.73 CD
	2HR	5.36 ± 1.12 B	1.61 ± 0.29 DE	2.63 ± 0.14 CDE	5.16 ± 0.43 B	2.78 ± 0.27 CDE
	4HR	3.36 ± 0.36 C	2.29 ± 0.002 CDE	3.22 ± 0.28 C	13.90 ± 0.46 A	3.19 ± 0.26 C
	6HR	5.00 ± 0.35 B	2.12 ± 0.11 CDE	5.48 ± 0.14 B	1.56 ± 0.09 DE	0.00 F

	1HR	9.90 ± 0.58 BCD	2.80 ± 0.27 CD	2.51 ± 0.35 CD	9.27 ± 0.28 BCD	5.76 ± 0.29 CD
<i>Total isoflavone (mg/</i>	2HR	11.65 ± 0.41 BC	5.41 ± 0.12 CD	5.31 ± 0.08 CD	11.30 ± 0.23 BC	6.45 ± 0.29 CD
<i>g dry roots)</i>	4HR	10.70 ± 0.14 BCD	7.04 ± 0.02 CD	5.62 ± 0.12 CD	27.01 ± 0.28 A	18.79 ± 0.09 AB
	6HR	9.36 ± 0.22 BCD	7.49 ± 0.11 BCD	26.96 ± 0.08 A	2.49 ± 0.05 CD	0.13 ± 0.002 D

*Results are the mean of three replicates ± SD and different letters in the same isoflavone detected are significantly different at $P < 0.05$, using Tukey test.

Table S3. Effect of elicitors on the accumulation of major and total isoflavones (mg/ g dry plant) in two *T. pratense* hairy roots line. *

<i>Elicitor</i>	<i>HR line</i>	<i>Days</i>	<i>Daidzein</i>	<i>Genistein</i>	<i>Formononetin</i>	<i>Biochanin A</i>	<i>Total isoflavones</i>
Sucrose 10 g/L	4 HR	3	4.975 ± 0.159 BCD	0.159 ± 0.002 K	4.049 ± 0.180 CD	1.846 ± 0.121 FGHIJK	11.029 ± 0.116 DEFGHIJ
		3.5	2.942 ± 0.059 BCDEFGH	0.110 ± 0.001 K	2.053 ± 0.026 CD	0.708 ± 0.013 JKLM	5.814 ± 0.025 GHIJKL
		4	3.850 ± 0.001 BCDEF	0.167 ± 0.005 JK	2.294 ± 0.049 CD	0.882 ± 0.003 IJKLM	7.192 ± 0.014 FGHIJKL
		4.5	1.906 ± 0.052 EFGHI	0.625 ± 0.001 DEFG	2.263 ± 0.010 CD	1.388 ± 0.027 GHIJKLM	6.182 ± 0.022 FGHIJKL
		5	1.360 ± 0.010 FGHI	0.448 ± 0.004 FGHIJK	1.268 ± 0.008 D	1.109 ± 0.012 HIJKLM	4.185 ± 0.008 HIJKL
		8	2.583 ± 0.036 CDEFGHI	0.869 ± 0.012 CDE	0.547 ± 0.003 D	0.937 ± 0.063 HIJKLM	4.937 ± 0.029 HIJKL
		11	0.559 ± 0.002 HI	0.533 ± 0.003 EFGHI	0.400 ± 0.006 D	1.707 ± 0.144 GHIJK	3.199 ± 0.039 IJKL
	14	0.313 ± 0.027 HI	0.356 ± 0.004 FGHIJK	0.548 ± 0.001 D	1.312 ± 0.119 GHIJKLM	2.529 ± 0.038 JKL	
	8 HR	3	0.988 ± 0.004 GHI	0.924 ± 0.003 CD	3.521 ± 0.024 CD	4.341 ± 0.026 BCD	9.774 ± 0.014 DEFGHIJK
		3.5	1.206 ± 0.007 FGHI	1.326 ± 0.005 B	3.015 ± 0.001 CD	4.373 ± 0.003 BCD	9.921 ± 0.004 DEFGHIJ
		4	2.479 ± 0.011 DEFGHI	2.525 ± 0.045 A	3.938 ± 0.004 CD	4.995 ± 0.034 BC	13.938 ± 0.024 CDEFG
		4.5	1.558 ± 0.015 EFGHI	1.380 ± 0.010 B	0.499 ± 0.028 D	0.795 ± 0.008 JKLM	4.232 ± 0.015 HIJKL
		5	1.057 ± 0.024 GHI	1.165 ± 0.010 BC	1.507 ± 0.055 D	2.247 ± 0.043 EFGHI	5.840 ± 0.033 GHIJKL
		8	1.128 ± 0.003 GHI	1.114 ± 0.025 BC	1.397 ± 0.006 D	3.175 ± 0.023 DEF	6.815 ± 0.014 FGHIJKL
4 HR		3	1.536 ± 0.305 EFGHI	0.404 ± 0.029 FGHIJK	17.011 ± 5.510 A	4.354 ± 0.740 BCD	23.306 ± 1.646 AB
	3.5	12.774 ± 1.332 A	0.652 ± 0.066 DEF	13.383 ± 1.156 AB	1.075 ± 0.199 HIJKLM	27.883 ± 0.688 A	

	4	12.465 ± 1.720 A	0.620 ± 0.061 DEFG	2.843 ± 0.432 CD	0.147 ± 0.076 LM	16.075 ± 0.572 BCDE
	4.5	1.895 ± 0.031 EFGHI	0.513 ± 0.031 FGHIJ	15.184 ± 5.289 A	4.217 ± 0.673 CD	21.808 ± 1.561 ABC
Salicylic acid 10 mg/L	3	4.184 ± 0.164 BCDE	0.687 ± 0.014 DEF	0.604 ± 0.088 D	0.157 ± 0.055 LM	5.461 ± 0.080 GHIJKL
	3.5	1.937 ± 0.298 EFGHI	0.395 ± 0.009 FGHIJK	14.763 ± 0.879 A	4.114 ± 0.302 CD	21.209 ± 0.372 ABC
	4	5.397 ± 0.271 B	0.220 ± 0.028 HIJK	7.973 ± 1.031 BC	1.803 ± 0.239 FGHIJK	14.943 ± 0.392 BCDEF
	4.5	5.002 ± 1.103 BCD	0.612 ± 0.150 DEFG	2.054 ± 0.467 CD	0.418 ± 0.085 KLM	8.086 ± 0.451 EFGHIJKL
	3	5.171 ± 0.010 BC	0.301 ± 0.028 GHIJK	3.526 ± 0.118 CD	2.642 ± 0.092 EFG	11.639 ± 0.062 DEFGHI
	3.5	4.099 ± 0.064 BCDE	0.208 ± 0.008 HIJK	1.752 ± 0.033 CD	1.503 ± 0.112 GHIJKL	7.563 ± 0.054 EFGHIJKL
	4	5.229 ± 1.089 BC	0.239 ± 0.011 HIJK	2.243 ± 0.355 CD	1.276 ± 0.183 GHIJKLM	8.987 ± 0.410 DEFGHIJKL
	4.5	4.167 ± 0.153 BCDE	1.153 ± 0.019 BC	1.652 ± 0.090 CD	1.513 ± 0.076 GHIJKL	8.485 ± 0.084 DEFGHIJKL
8 HR	3	3.618 ± 0.617 BCDEFG	0.410 ± 0.061 FGHIJK	5.867 ± 0.866 CD	7.279 ± 1.347 A	17.174 ± 0.723 BCD
	3.5	1.384 ± 0.003 FGHI	0.202 ± 0.002 HIJK	2.444 ± 0.044 CD	3.463 ± 0.002 DE	7.492 ± 0.013 EFGHIJKL
	4	2.208 ± 0.073 EFGHI	0.123 ± 0.005 K	1.556 ± 0.016 D	2.036 ± 0.034 EFGHIJ	5.923 ± 0.032 GHIJKL
	4.5	2.327 ± 0.561 DEFGHI	0.548 ± 0.355 EFGH	1.220 ± 0.133 D	1.335 ± 0.170 GHIJKLM	5.181 ± 0.306 GHIJKL
Salicylic acid 30 mg/L	3	1.935 ± 0.077 EFGHI	0.599 ± 0.030 DEFG	1.379 ± 0.085 D	1.324 ± 0.086 GHIJKLM	5.237 ± 0.070 GHIJKL
	3.5	1.318 ± 0.032 FGHI	0.167 ± 0.008 JK	0.886 ± 0.022 D	0.815 ± 0.019 IJKLM	3.186 ± 0.020 IJKL
	4	0.375 ± 0.012 HI	0.397 ± 0.003 FGHIJK	0.013 ± 0.006 D	0.171 ± 0.0002 LM	0.956 ± 0.005 KL
	4.5	0.172 ± 0.016 I	0.155 ± 0.001 K	0.178 ± 0.007 D	0.042 ± 0.005 M	0.369 ± 0.007 L

	3	0.836 ± 0.011 HI	0.623 ± 0.015 DEFG	0.294 ± 0.010 D	0.564 ± 0.047 KLM	2.307 ± 0.021 JKL
8 HR	3.5	1.626 ± 0.020 EFGHI	0.165 ± 0.014 JK	4.666 ± 0.010 CD	5.740 ± 0.012 B	12.198 ± 0.014 DEFGH
	4	1.726 ± 0.019 EFGHI	0.198 ± 0.007 IJK	1.889 ± 0.058 CD	2.355 ± 0.071 EFGH	6.167 ± 0.039 FGHIJKL
	4.5	1.112 ± 0.030 GHI	0.138 ± 0.001 K	0.631 ± 0.006 D	0.896 ± 0.007 IJKLM	2.776 ± 0.011 JKL

*Results are the mean of three replicates ± SD and different letters in the same columns are significantly different at $P < 0.05$, using Tukey test.

Tables

Table 1. Isoflavonoids identified in *T. pratense* hairy root cultures lines by HPLC-ESI-MS/MS.

Cpd	t _R ^a	Aglyc MH ⁺ ^b	(m/z) ^c	Product ions ^d	Identification ^e
1	5.68	350	350	-	^g
2	5.84	350	350	-	^g
3	8.01	315	477	162	3',7-Di-O-Methylorobol (5-Hydroxy-3-(4-hydroxy-3-methoxyphenyl)-7-methoxy-4H-chromen-4-one) Hexose
4	8.23	285	447	162	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin Hexose
5	9.3	315	563	248	3',7-Di-O-Methylorobol (5Hydroxy-3-(4-hydroxy-3-methoxyphenyl)-7-methoxy-4H-chromen-4-one) Malonyl Hexose
6	9.75	285	533	248	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin - Malonyl Hexose
7	10.48	301	549	248	Pratensein/ 3 O Methylorobol (5,7-dihydroxy-3-(4-hydroxy-3-methoxyphenyl)-4Hchromen-4one) Malonyl Hexose
8	11.02	331	331	-	^g
9	11.14	269	431	162	Formononetin - hexose
10	11.6	283	531	248	Pseudobaptigenin- Malonyl hexose
	11.66	285	533	248	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin - Malonyl Hexose

11	11.71	315	315	-	3',7-Di-O-Methylorobol (5Hydroxy-3-(4-hydroxy-3-methoxyphenyl)-7-methoxy-4H-chromen-4-one)
12	11.94	269	517	248	Formononetine -Malonyl Hexose
13	12.05	283	531	248	Pseudobaptigenin- Malonyl Hexose
14	12.33	285	285	-	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin
15	12.41	269	517	248	Formononetin -Malonyl Hexose
16	13.26	285	533	248	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin - Malonyl Hexose
17	13.52	299	547	162	Irilone/Afrormosine-Malonyl Hexose
18	13.54	301	549	248	Pratensein/ 3 O Methylorobol (5,7-dihydroxy-3-(4-hydroxy-3-methoxyphenyl)-4Hchromen-4one) Malonyl Hexose
19	14.41	285	533	248	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin - Malonyl Hexose
20	14.58	301	301	-	Pratensein/ 3 O Methylorobol (5,7-dihydroxy-3-(4-hydroxy-3-methoxyphenyl)-4Hchromen-4one)
21	15.51	283	283	-	Pseudobaptigenin
22	15.92	269	269	-	Formononetin
23	17.29	299	299		Irilone/Afrormosine
24	18.29	307	365	-	g
25	18.51	285	285		Biochanin A
26	19.36	269	372	103	Formononetine Malonyl

27	19.66	417	417	-	Daidzein
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^a rT: retention time in minutes; **Cpd**: Compound.

^b Aglycone MH+: molecular ion weight provided by ESI-MS in a positive mode after the loss of the sugar molecule.

^c m/z: [M+H]⁺: molecular ion weight provided by ESI-MS in a positive mode.

^d Product ions: fragment obtained by MS/MS with the collision energy.

^e Identification: suggested structure of the compound based on rT and the m/z confirmed with a standard (**data in bold**) or from MS and MS/MS spectra deduction.

^f References: studies with the identification of the compound.

^g Compound not identified.

Figure Captions:

Fig. 1 Isoflavonoids biosynthetic pathway showing the general phenylpropanoid metabolic pathway and the isoflavone pathway with the most known molecules: daidzein (**a**), genistein (**b**), formononetin (**c**), biochanin A (**d**) and the estrogen 17 β -estradiol (**e**) for molecular comparison. The majority of isoflavone molecules are formed by a split pathway, according to their key intermediates. Genistein originates through naringenin and subsequently, via isoflavone 4'-*O*-methyltransferase enzyme generates biochanin A. For daidzein, the intermediate is liquiritigenin and with the action of the same isoflavone 4'-*O*-methyltransferase it gives formononetin. Adapted from (Du et al. 2010; Kanehisa et al. 2017)

Fig. 2 Root growth (mm) after seven days of culture in MS medium containing different concentrations of (a) sucrose + agar or phytigel, (b) salicylic acid, (c) yeast extract and (d) flagellin 22. Results are the mean of three replicates \pm SD and different letters in the same treatment are significantly different at $P < 0.05$ (Tukey test). The red bar represents the root growth of control conditions (sucrose 10 g/L with agar)

Fig. 3 Isoflavone content (mg /g dry plants) of *T. pratense* seedlings cultivated in medium with agar or phytigel as gelling agent (different concentration) and with elicitors at different concentrations. Seedlings were grown for seven days following germination on these different media before isoflavonoid content measurement. Panel (a) sucrose + phytigel or agar, panel (b) salicylic acid, panel (c) yeast extract and panel (d) flagellin 22. Results are the mean of three replicates \pm SD and different letters in the same treatment are significantly different at $P < 0.05$ (Tukey test). The red bar represents the isoflavone content in control conditions (sucrose 10 g/L with agar, panel a)

Fig. 4 *T. pratense* *A. rhizogenes* induced hairy root production. (a) Seedlings 10 days after infection. (b) Roots transferred to the medium without growth regulators, 30 days after the induction. (c) Development of the roots after 1 week on solid medium. (d) Transfer to liquid medium, 50 days after infection. (e) and (f) Hairy root after one week in liquid medium (above and below of the bottle). (g) and (h) The hairy root culture after 3 weeks in liquid medium

Fig. 5 Graphic representation of the *T. pratense* hairy root line isoflavonoid profiles represented as the percentage of the total flavonoid content. Each isoflavonoid compound is represented by a different colour and the percentage of the molecule is indicated in the corresponding part of the bar. The colour code is given on the right part of the figure. Note that each line has a specific isoflavonoid composition. Values below 3 % were removed from the graph representation

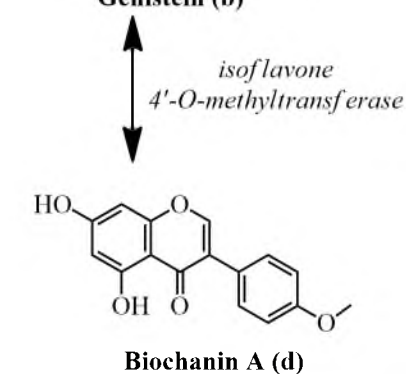
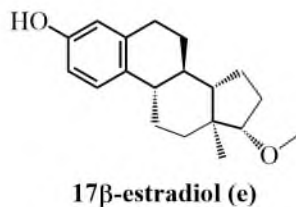
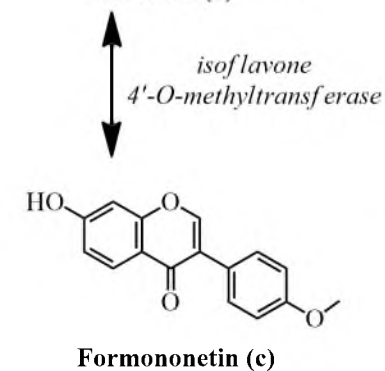
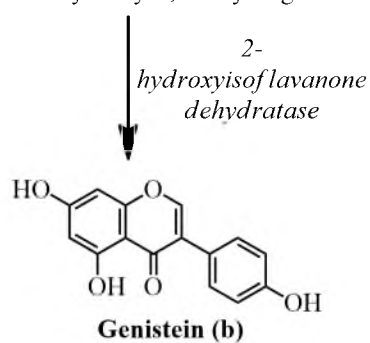
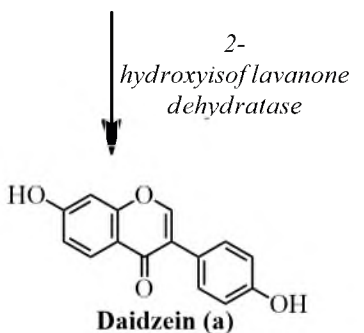
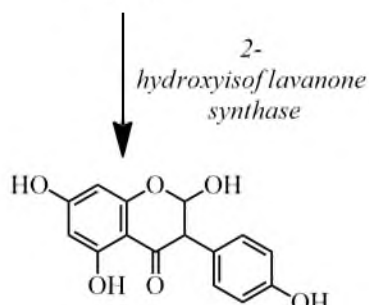
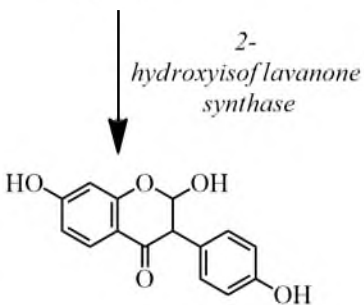
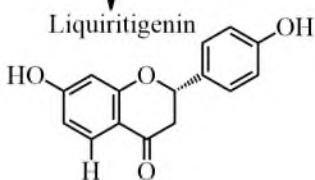
Fig. 6 Isoflavones content of the *T. pratense* HR lines 1HR, 2HR, 4HR and 6HR during the first 5 months of culture. (a) Daidzein; (b) Genistein; (c) Formononetin and (d) Biochanin A contents. The total isoflavonoid production is given in (e). The results are the mean of three replicates \pm SD and different letters in the same isoflavone analysis are significantly different at $P < 0.05$ (Tukey test). The colour code is indicated on the right side of the last panel

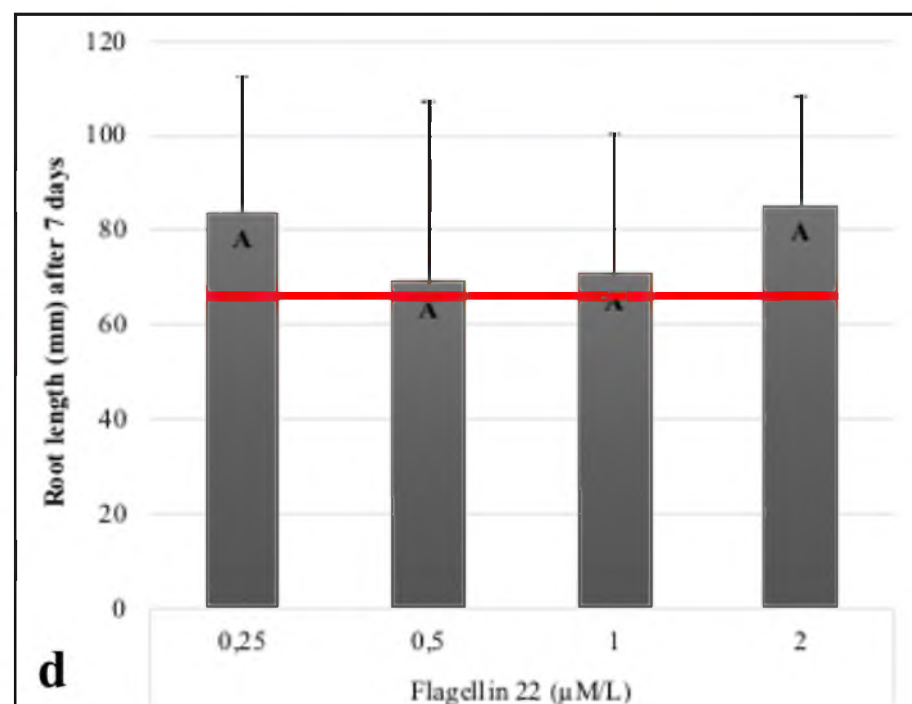
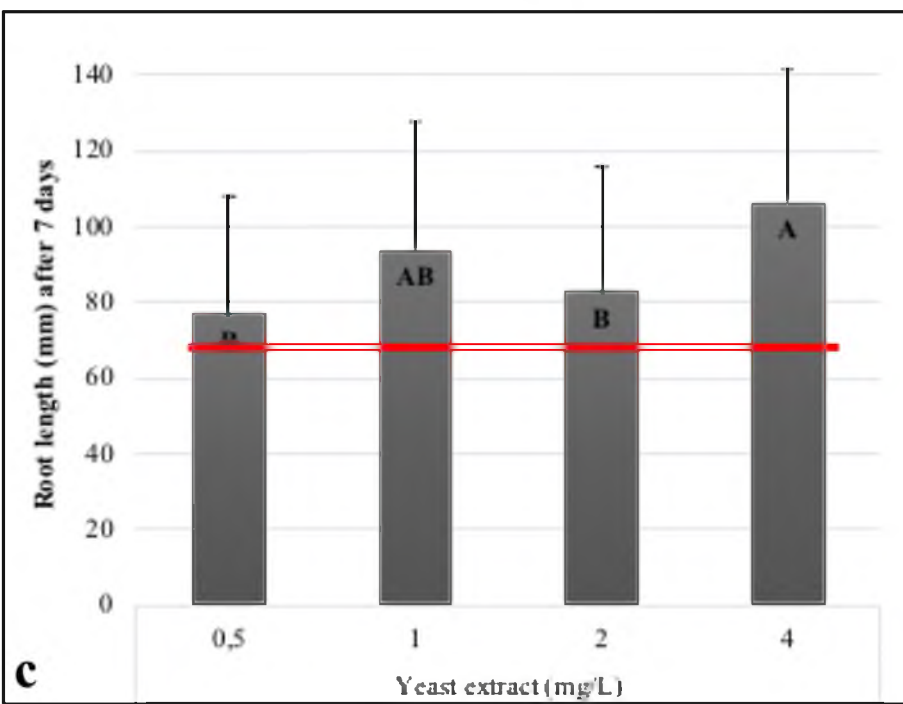
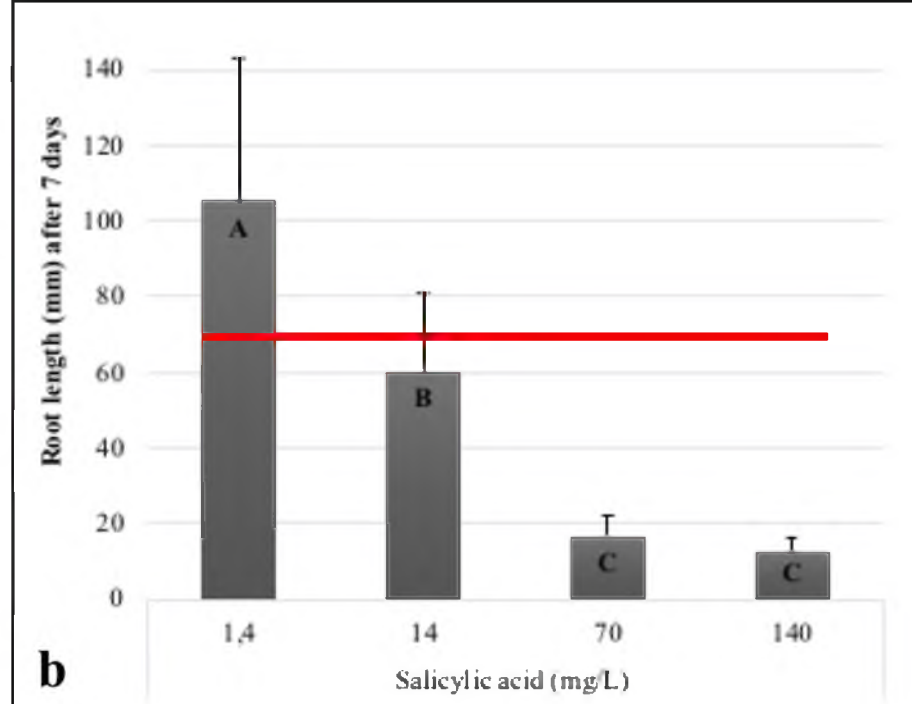
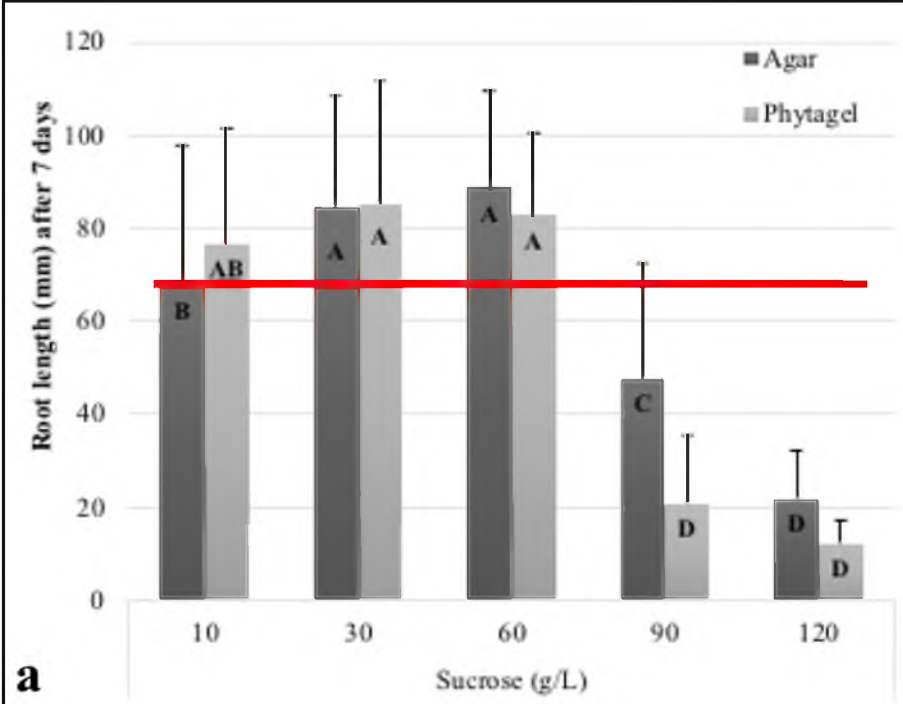
Fig. 7 Effect of sucrose 10 g/L (control, a-b) and 60 g/L (c-d), salicylic acid 10 g/L (e-f) and 30 mg/L (g-h) on the accumulation of major and total isoflavones *T. pratense* hairy roots. Line 4HR (a-c-e-g) and line 8HR (b-d-e-h). Results are the mean of three replicates \pm SD and different letters in the same isoflavone detected are significantly different at $P < 0.05$ (Tukey test). The time is represented in days

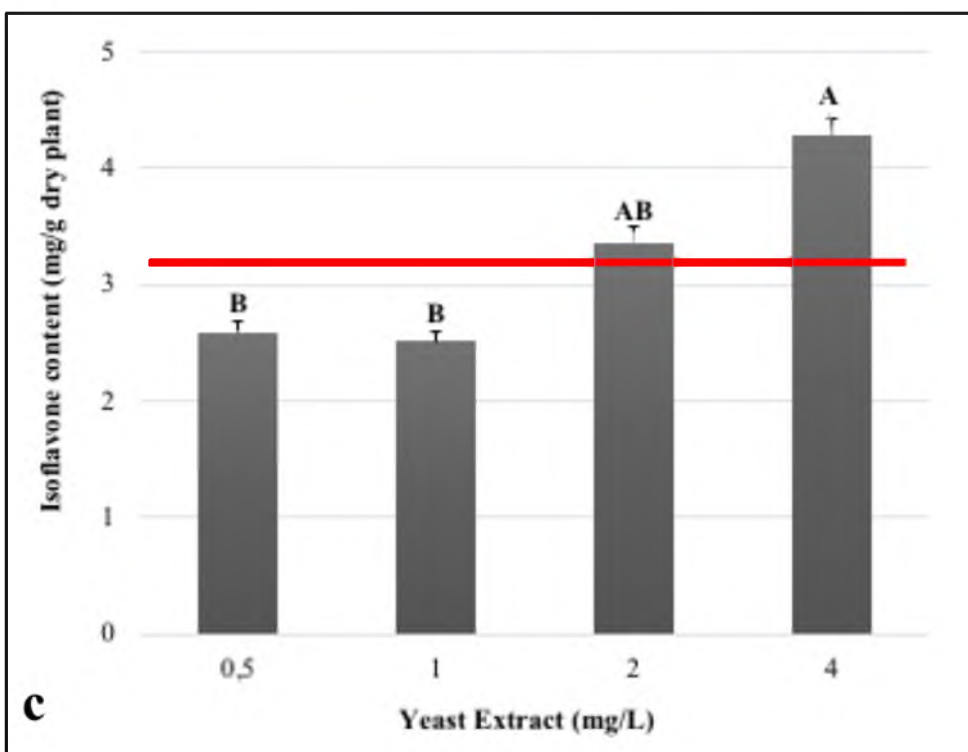
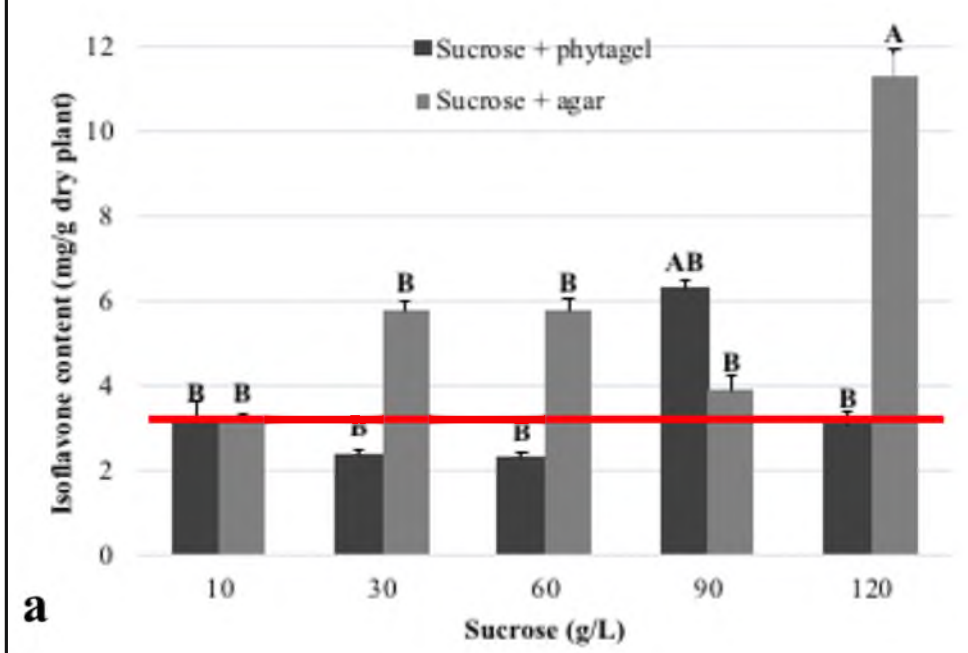
Fig. S1 Colour modification in the *T. pratense* plantlets flavonoid extracts cultivated in presence of Sucrose plus phytigel as gelling agent. From left to right extracts of cultures grown on 10, 30, 60, 90 and 120 g/L of sucrose

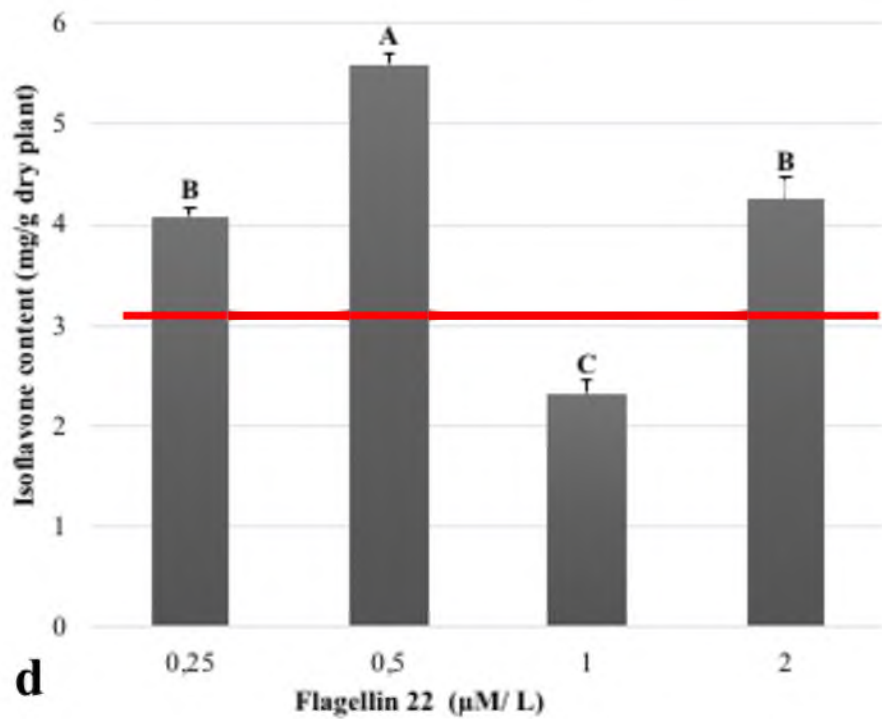
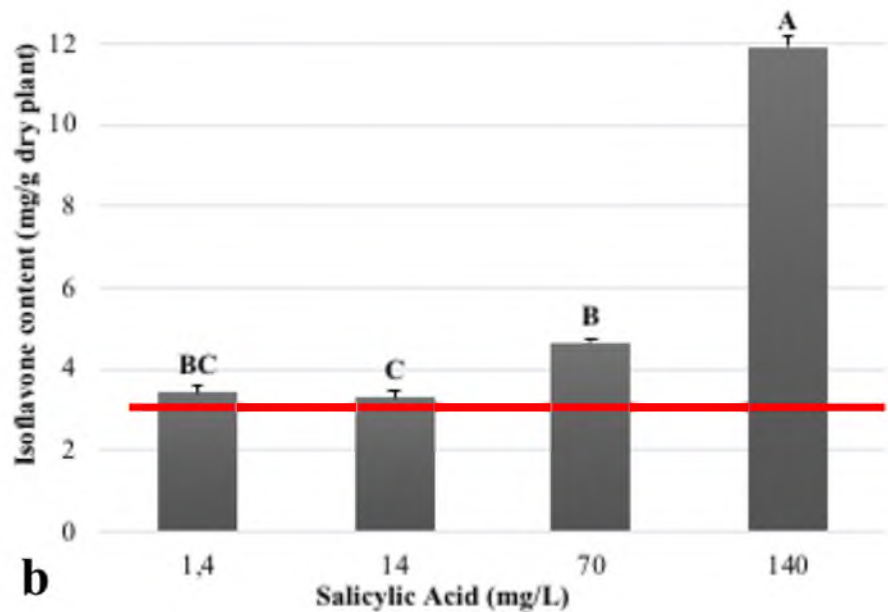
General Phenylpropanoid metabolic pathway

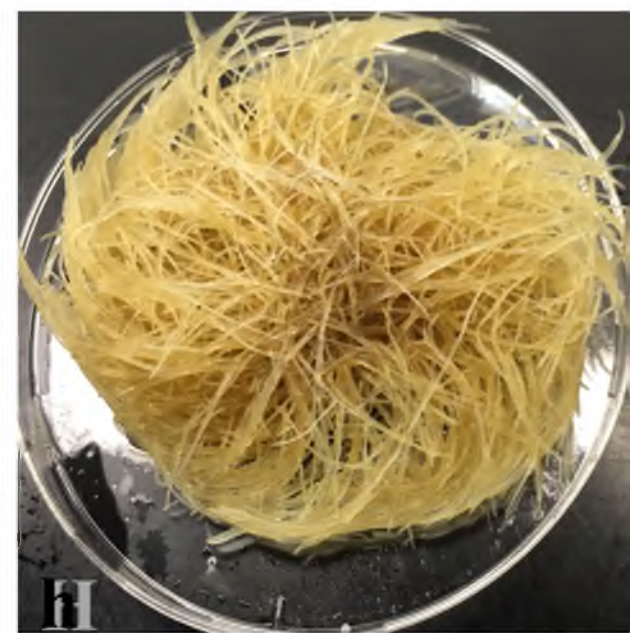
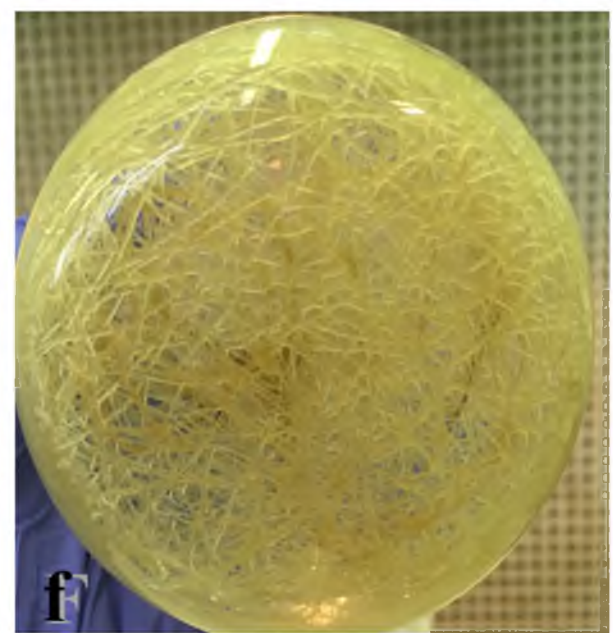
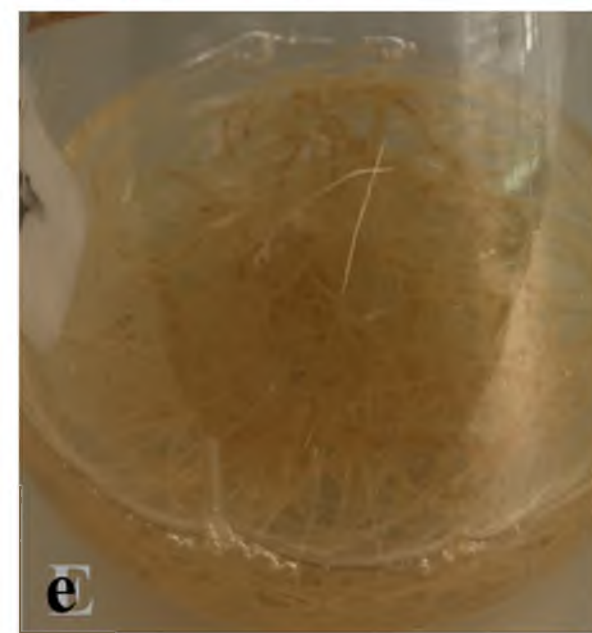
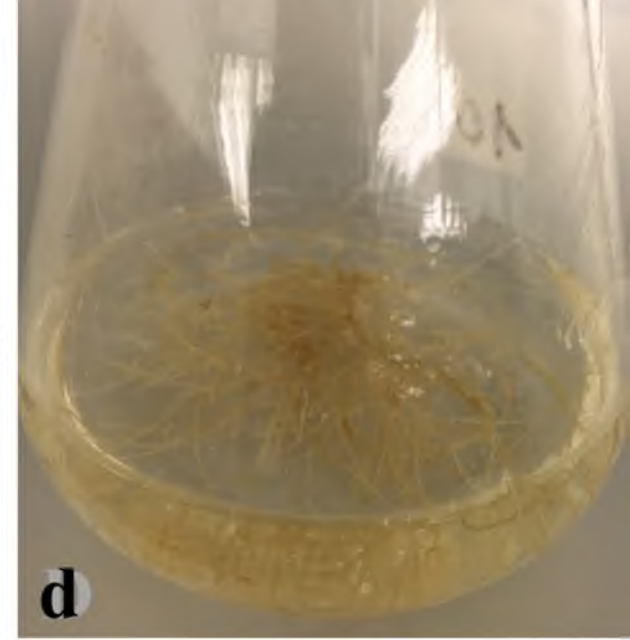
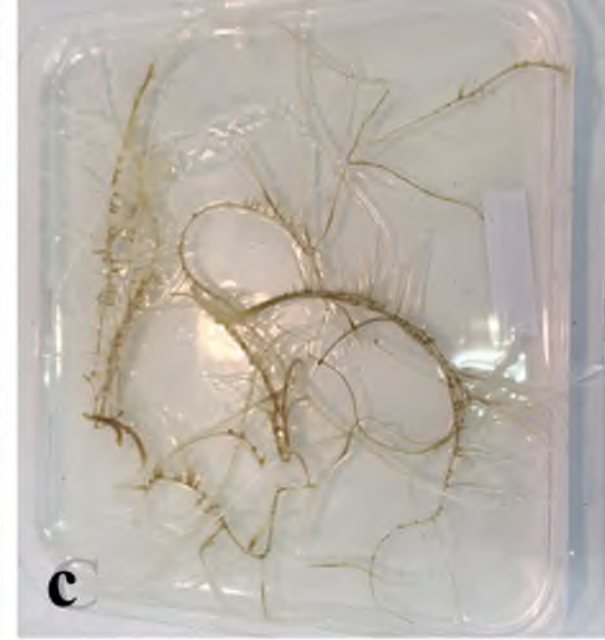
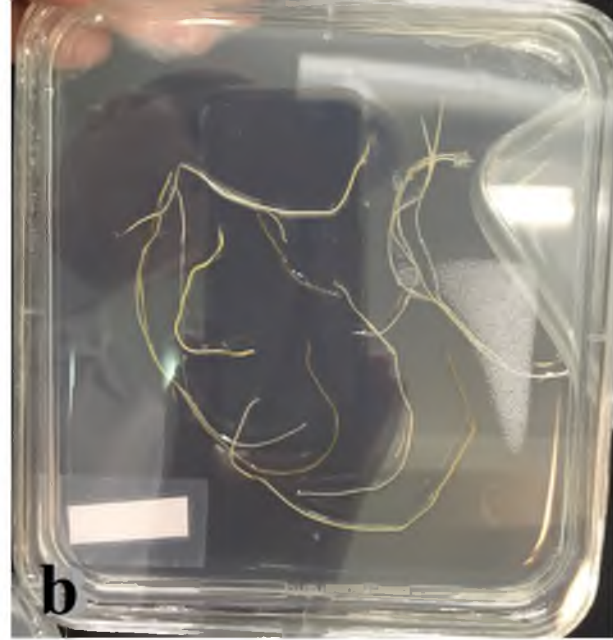
Flavonoid biosynthesis

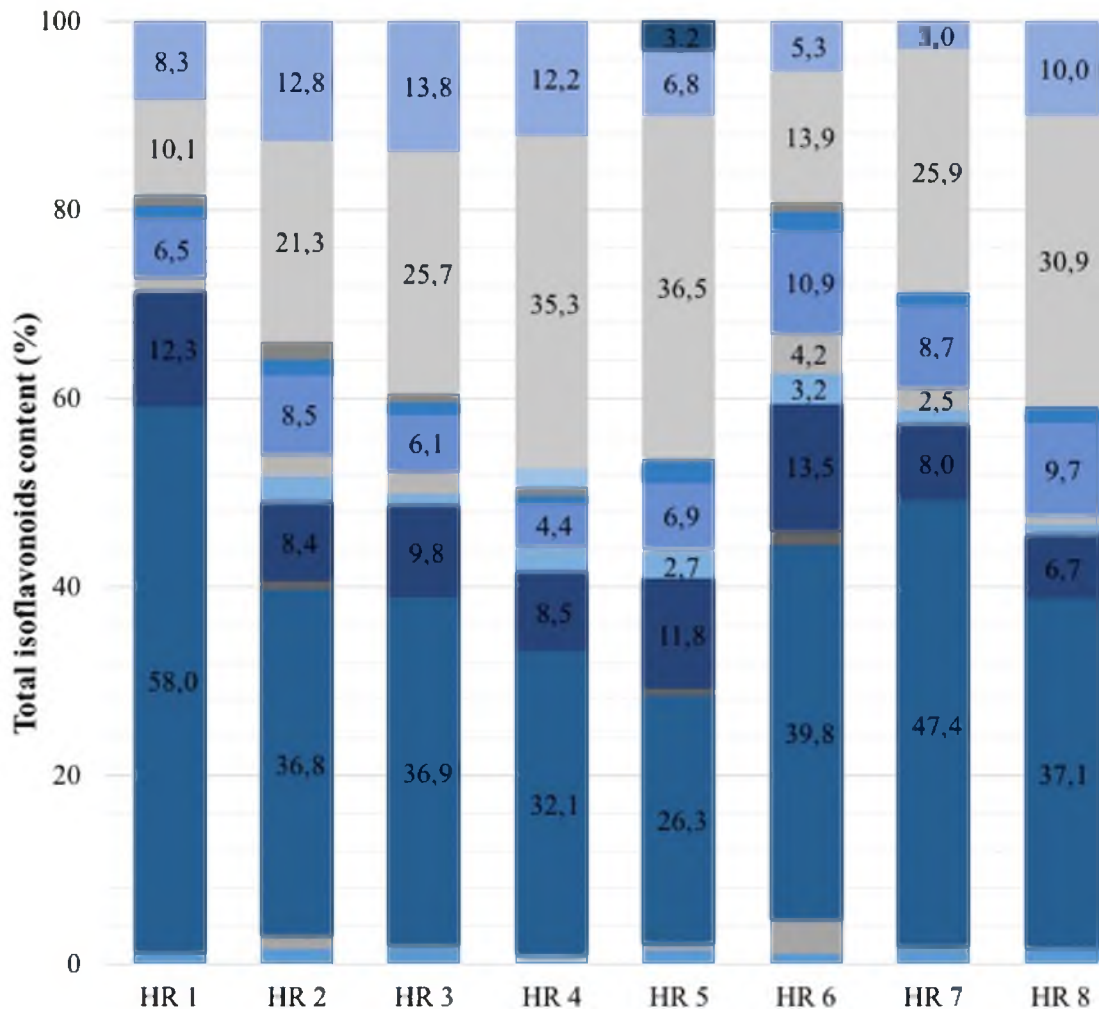












Hairy root lines

c

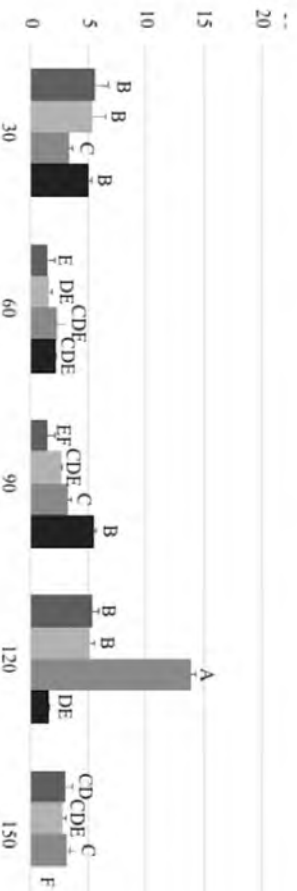
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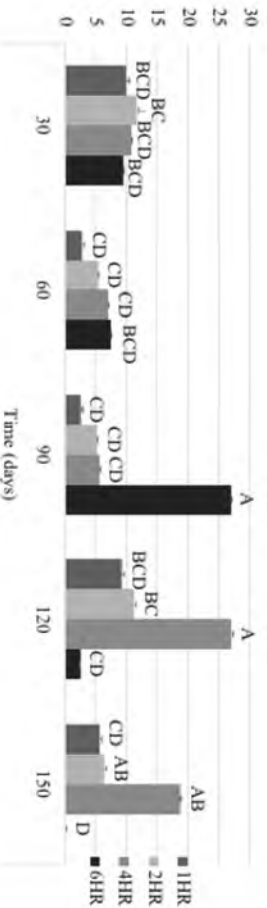
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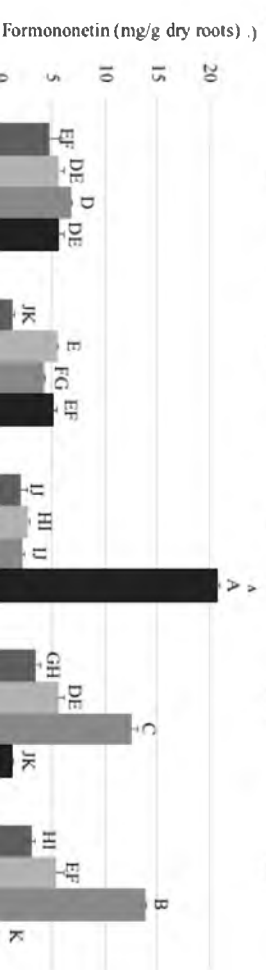
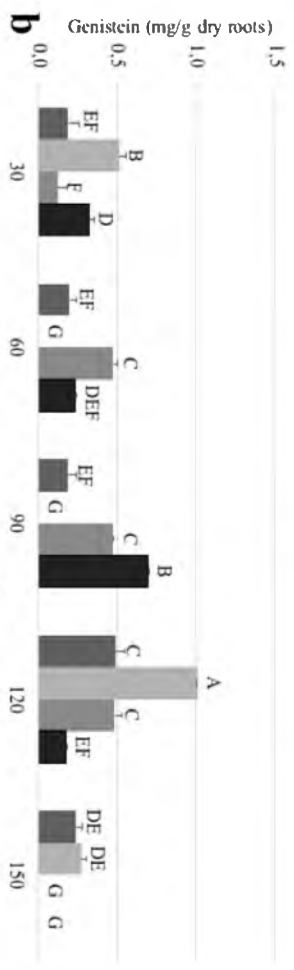
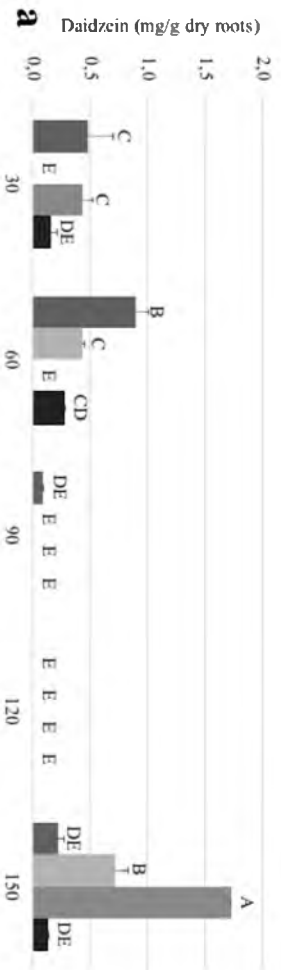
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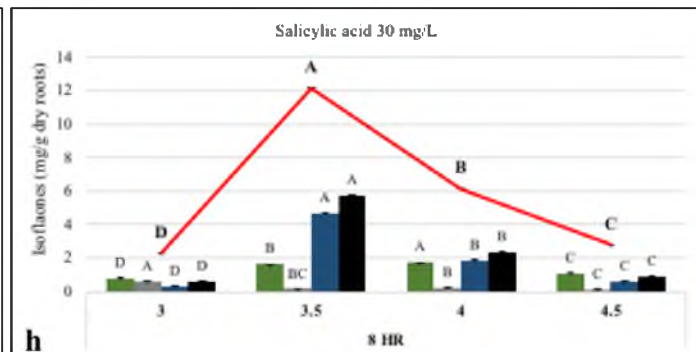
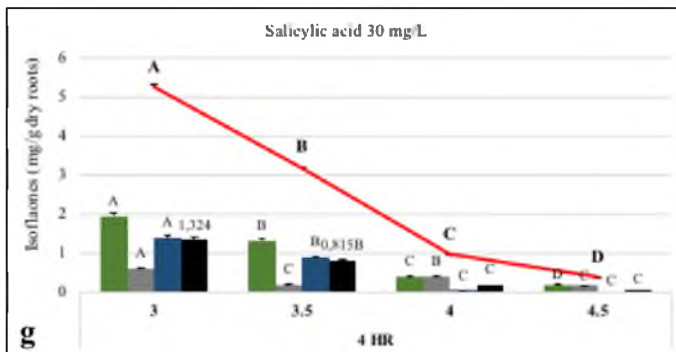
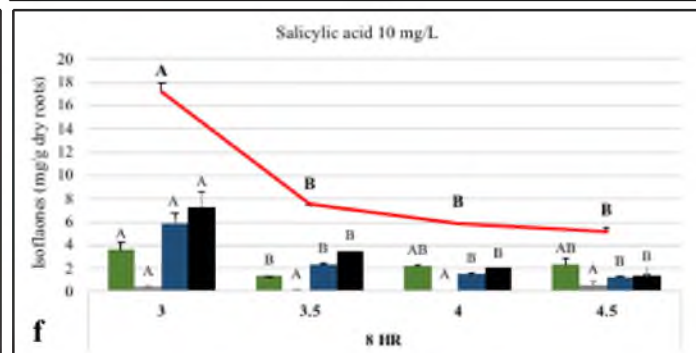
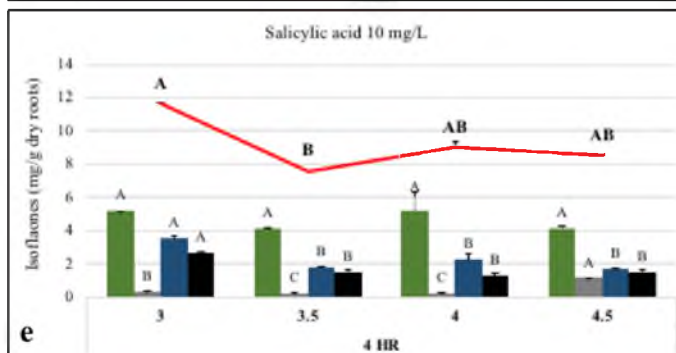
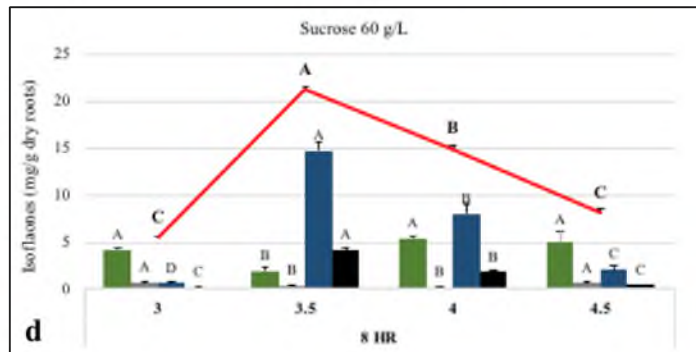
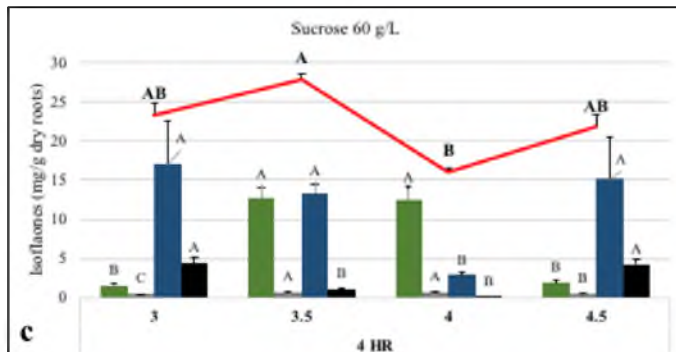
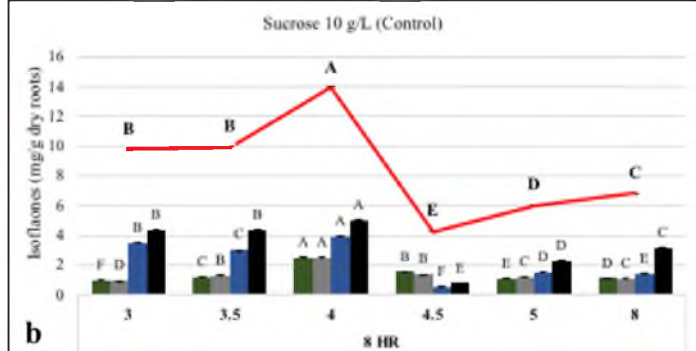
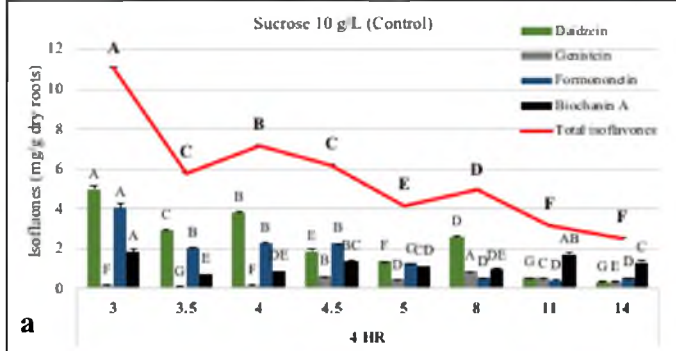
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d Biochanin A (mg/g dry roots)**e**

Total isoflavonoids content (mg/g dry plant)







510P

①

530P

①

560P

③

590P

①

5120P

③