Enhanced production of falcarinol-type polyacetylenes in hairy roots of cultivated carrot (*Daucus carota* subsp. *sativus*)

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Short Report

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Abstract

Polyacetylenes (PAs) are a large group of bioactive phytochemicals, which are primarily produced by higher plants of the families Apiaceae and Araliaceae. Especially aliphatic C_{17}-polyacetylenes of the falcarinol-type such as falcarinol (FaOH) and falcarindiol (FaDOH) are known for their numerous positive effects on human health. In this study we investigate the potential of carrot hairy root cultures for production of PAs. Three individual plants of seven differently coloured carrot cultivars were used for the development of hairy root cultures by transformation of root discs with the wild-type *Rhizobium rhizogenes* strain 15834. A total of 51 individual hairy root (HR) lines were obtained and quantitatively analysed together with root, petiole and leaf tissue samples for FaOH and FaDOH. Among the five tissues sampled from the donor plants, root periderm samples generally exhibited the highest PA levels with FaDOH as prevailing PA and large differences between cultivars. In comparison to periderm tissue, FaOH levels were highly increased in HR lines of all cultivars. In contrast, FaDOH levels were not significantly altered. Considering the low to moderate PA concentration in root and leaf tissues of the orange cultivars there was an up to more than 10-fold increase of the FaOH concentration in HRs of these genotypes. Within this study a reproducible method for *Rrhi*-mediated transformation of carrot root discs was applied which provides an efficient tool to assess the function of candidate genes involved in the biosynthesis of key PAs in carrot but might be used in future also for the large-scale production of falcarinol-type PAs.

Introduction

Rhizobium rhizogenes (*Rrhi*), formerly known as *Agrobacterium rhizogenes* is a soil bacterium that induces the hairy root disease in plants by transferring its DNA to plant cells and thereby altering the plant root architecture. Hairy roots (HRs) have been recognized as a promising *in vitro* culture system with a high capacity to produce specialized plant metabolites (for comprehensive overview, see Malik 2018). Wild-type *Rrhi* strains have been used for transformation of medicinal plants, with the aim to produce phytochemicals of potentially pharmaceutical importance (Guillon et al. 2008). In addition to *Rrhi* wild strains, genetically modified strains with altered Ri plasmids and/or additional binary vectors have been used widely in plant research and biotechnology. In the past three decades *Rrhi*-based transformation systems helped to elucidate physiological processes and biosynthetic pathways by functional gene studies. In this context, HR-based genome editing techniques such as CRISPR/Cas have been used in several plant species such as *Salvia* (Li et al. 2017), *Eucalyptus* (Dai et al. 2020) and *Medicago* (Zhang et al. 2020).

The cultivated carrot (*Daucus carota* ssp. *sativus)* is one of the most important root vegetable crops grown worldwide. Carrots provide not only one of the richest sources of the vitamin A precursor β-carotene, but contain many other bioactive phytochemicals including anthocyanins, phenylpropanoids, polyacetylenes and terpenoids. Several Apiacae species were investigated in the past for production of plant natural products in HR culture systems (Baranski 2008). Starting with pioneer work in early 1980s (Chilton et al. 1982; Willmitzer et al. 1982) several HR-based studies were reported for *D. carota* (Kim and Yoo 1996; Ridgway et al. 2004). Recently, HR cultures derived from dark-purple carrots were shown to be suited for the *in vitro* production of anthocyanins and phenolic compounds (Barba-Espin et al. 2020). An efficient *Rrhi*-based carrot transformation protocol including subsequent plant regeneration was developed by Baranski et al. (2006), and a modified version of this protocol was used for CRISPR/Cas9-mediated genome editing in carrots (Dunemann et al. 2019).

Polyacetylenes (PAs) are a large group of bioactive phytochemicals, which are primarily produced by higher plants of the families Apiaceae and Araliaceae both belonging to the order Apiales. Also they are common in Asteraceae family members (Dawid et al. 2015). Among the more than 1.400 known PAs (Christensen and Brandt 2006), a subset of 12 structurally related PAs were isolated from *D. carota* (Dawid et al. 2015). These PAs are found in common vegetables and herbs of the Apiaceae family such as carrots, parsnip, fennel, celery, and parsley, with the C_{17}-PAs falcarinol (FaOH) and falcarindiol (FaDOH) as the main PAs (Czepa and Hoffmann 2003, Kidmose et al. 2004). Both FaOH and FaDOH are known to have a major impact on bitter off-taste in carrots (Schmiech et al. 2008; Kreutzmann et al. 2008). A series of *in*

vitro and *in vivo* studies showed that in particular the aliphatic C_{17}-PAs of the falcarinol-type exhibit potent anti-microbial, anti-inflammatory, and anti-cancer effects (Zidorn et al. 2005; Christensen and Brandt 2006; Christensen 2011; Dawid et al. 2015; Christensen 2020). For instance, falcarinol-type PAs may function as inhibitors of the breast cancer resistance protein BCRP/ABCG2, indicating their prospective use as agents for cancer chemotherapy (Tan et al. 2014). Rat feeding experiments with purified FaOH and FaDOH suggest preventing effects of these PAs on the development of colorectal cancer (Kobaek-Larsen et al. 2019).

In this study we describe the usage of a *Rrhi*-based transformation system for generation of HRs derived from orange, yellow and purple carrots. Levels of two major PAs FaOH and FaDOH were quantified in HRs and leaves and roots of the original carrot donors. Moreover, we elucidated the potential of HR cultures to produce sufficient amounts of these PAs and show that especially FaOH is strongly increased in HRs of orange carrots compared to peridermal root tissue.

Material And Methods

Plant Material

Seven carrot cultivars (*D. carota* subsp. *sativus*) with differently coloured roots were used in this study: 'Anthonina' (AN) - purple, 'Deep Purple' (DP) - purple, 'Yellowstone' (YS) - yellow, '710015' (71) - yellow, 'Rotin' (RO) - orange, 'Lange Rote Stumpfe ohne Herz' (LR) - orange, 'Shinsuu Senkou Oonaga' (SO) - orange. Seeds were obtained from the JKI carrot seed collection kept by Dr. Thomas Nothnagel. Three plants of each cultivar were sown and cultivated in 17 cm plastic pots in a standard soil mixture (sand/humus, 3/1 (v/v)) under controlled greenhouse conditions (16 h photoperiod, 20 – 25°C) until harvest about 15 weeks after sowing. Tap roots were washed to remove adhering soil and blotted dry. Periderm samples were obtained from a 3-cm section from the middle of each carrot root using a peeler. Afterwards, roots were latitudinally halved and an 8 mm thick slice was removed from each root half. Using a scalpel phloem and xylem samples were prepared from both slices. The remaining root material was sterilized and used for transformation. Petiole samples were obtained by cutting a 5-cm section from the middle of three petioles. Leaf tissue was sampled from younger leaflets. Plant material was shock frozen in liquid nitrogen immediately after harvest and stored at -80°C until freeze-drying.

Transformation of carrot with *Rhizobium rhizogenes*

Pieces of tap roots remaining after PA sampling were surface-sterilized via immersion in 70% ethanol for 5 min followed by a 20 min treatment with 4% sodium hypochlorite supplemented with 0.1% Tween 20. Each carrot root was sliced into 0.5 – 0.8 cm thick discs and dead tissue of the outer edge of the discs was removed. The apical (shoot-averted) side was labeled with a sterile toothpick, and the carrot discs were placed (apical side up) onto water agar (2% agar in water; Duchefa, Haarlem, Netherlands). Wild-type *Rrhi* strain ATCC 15834 (Lippincott et al. 1973) was used to grow bacterial inocula in dark conditions at 28°C for about 24 h in liquid CPY medium (bacto-yeast extract 0.1%, peptone 0.5%, saccharose 0.5%, bacto-agar 20 g L^{-1}, $MgSO_4x7H_2O$ 200 mM) on a rotary shaker (180 rpm). About 100 µl of the bacterial suspension (OD_{600} of 0.6 - 0.8) was spread along the cambial ring on the apical side of the root discs. After co-cultivation for seven days on 2% water agar medium the carrot discs were placed for up to five weeks on 2% water agar supplemented with 200 mg L^{-1} cefotaxime (Duchefa, Haarlem, Netherlands) and 100 mg L^{-1} carbenicillin (Roth, Karlsruhe, Germany) to eliminate the bacteria. HRs started to regenerate generally after two weeks and were harvested weekly alongside with a small piece of original root tissue. Regenerated HRs were placed in small petridishes (6 cm diameter) on hormone-free half-concentrated MS (Murashige & Skoog) medium with 200 mg L^{-1} cefotaxime and 100 mg L^{-1} carbenicillin (½ MS-CC) and cultured in the dark at 22°C. HR cultures developing from single excised roots that originated unambigously from different regions of the root disc were considered to have an independent origin of regeneration. They were placed in a small single petridish and were designated as 'hairy-root line' (HR line). About four weeks later the propagating HRs were subcultured on the same culture medium. After further one to two subcultures in small plates, the HRs were placed on filter

paper located on the surface of a ½ MS culture medium without antibiotics in 9 cm petri dishes. Four weeks later the HR lines were harvested and frozen immediately at -80°C until chemical analysis.

In order to verify the transgenic character of the HRs and to prove that they were free of contaminating *Rrhi* bacteria we performed PCR analyses. For DNA isolation, 50 - 100 mg fresh hairy roots were snap frozen in liquid N_2 and grinded using a swing mill to extract total genomic DNA with the innuPREP Plant DNA kit (Analytik Jena, Jena, Germany). The DNA content was measured via NanoDrop device (Thermo Fisher). To check the transgenic status of the HR lines, we used PCR primer for the *rolC* gene from the Ri plasmid of *Rrhi,* which are diagnostic for the integration of the T-DNA into the plant genome (for primer information and PCR conditions, see Medina-Bolivar et al. 2007). To prove that the presence of the *rolC* gene is due to its integration into the genome of *D. carota* and not due to contamination with *Rrhi* we performed PCR analysis with primer for the *Rhizobium virD2* gene which is not transmitted into the plant genome (Medina-Bolivar et al. 2007).

Sample preparation for polyacetylene analysis

Deep-frozen plant material (leaf, petiole, periderm, phloem, xylem, hairy roots) was freeze-dried for four days (Christ Gamma 1–16 LSC, condenser temperature − 50°C, pressure 0.04 mbar). Depending on the quantity, dried plant material was homogenized in 1.5-ml or 5-ml polypropylene centrifuge tubes or in 10-ml polyethylene vials using steel balls (Ø 3–10 mm) and a mixer mill (Retsch MM 400, 30 Hz, 1–2 min). Homogenized plant material was precisely weighed [leaf and petiole tissue: (40 ± 1) mg; periderm, phloem, xylem, hairy root tissue: (20 ± 1) mg] into a 1.5-mL polypropylene centrifuge tube. After addition of three steel balls (Ø 3 mm), 50 µL internal standard solution (0.2 g L^{-1} *N*-vanillylnonamide in MeOH) and 300 µL acetone the mixture was homogenized using a mixer mill (Retsch MM400, 30 Hz, 60 s, room temperature). Afterwards, the sample was sonicated (ElmaSonic P, 37 kHz, 100 W, 5 min, 20°C) and shaken (2400 min^{-1}, 10 min, room temperature). After centrifugation (13000 *g*, 5 min, 22°C) a 200-µL aliquot of the supernatant was transferred into an HPLC vial with micro-insert and stored at 6°C until analysis.

Analysis of polyacetylenes by HPLC/DAD

Polyacetylene analyses were performed on an 1100 Series HPLC system (Agilent Technologies) comprising a degasser (G1322A), a binary pump (G1312A), an autosampler (G1329A), an autosampler thermostat (G1330A), a column compartment (G1316A) and a diode array detector (G1315A). Extracts (injection volume 2.5 µL) were separated on a Zorbax Eclipse XDB-C18 column (3 mm × 150 mm, 3.5 µm particle size, Agilent Technologies) using water and acetonitrile as eluent A and B, respectively. The following binary gradient program at a flow rate of 1 mL min^{-1} was used: 0-10 min, linear from 50 to 80 % B; 10-10.5 min, linear from 60 to 100 % B; 10.5-13 min, isocratic, 100 % B; 13-15 min, isocratic 50 % B. The column and autosampler temperature was maintained at 40°C and 6°C, respectively. The diode array detector response time was set at 0.2 s, the optical slit width at 4 nm. Polyacetylenes were detected at 196 nm with a spectral bandwidth of 4 nm, the internal standard *N*-vanillylnonamide at 204 nm with a spectral bandwidth of 4 nm. ChemStation software (version B.03.02) was applied for controlling the instrument, data acquisition and quantitative analysis. *N*-Vanillylnonanamide (t_R 2.53 min), FaDOH (t_R 5.09 min) and FaOH (t_R 9.48 min) were quantified based on peak area using external standard calibration method. Therefore, the following calibration curves were established : (i) *N*-vanillylnonamide: calibration range 1 – 600 ng, 14 points, linear regression model (y = m x), equal weighting, R^2 = 0.99987; (ii) FaDOH: calibration range 5 – 500 ng, 9 points, linear regression model (y = m x), equal weighting, R^2= 0.99996; (iii) FaDOH: calibration range 500 – 1000 ng, 6 points, logarithmic regression model (y = m ln(x) + b), equal weighting, R^2 = 0.99919; (iv) FaOH: calibration range 5 – 1000 ng, 14 points, linear regression model (y = m x), equal weighting, R^2 = 0.99970. Polyacetylene levels were corrected using the recovery rate of the internal standard. The average recovery rate of the internal standard was 101.3 % (standard deviation 4.6 %, n = 156).

Results And Discussion

Hairy root induction in seven differently coloured *D. carota* cultivars was accomplished by co-culture of carrot root disks and *Rrhi* wild strain 15834. Already 12 days after inoculation first HRs protruded from the upper surface of the root disks. HR cultures (HR lines) were established from single excised roots and, after selection for sufficient growth, used for PA analysis. To prove the presence of the T-region of the Ri plasmid and the integration of the *rol* (root loci) genes into the genome of *D. carota* we performed PCR analysis with *rolC* primer. The transgenic character of the HR lines was verified, and the *virD2* fragment indicating bacterial contamination was not detected in any HR line analyzed (PCR results not shown). A single transformation experiment based on the three individual tap roots of each carrot cultivar used for PA analysis resulted in a total number of 51 well growing HR lines at the end of the propagation process (Table 1). Cultivars DP, YS, and LR totally yielded the desired three lines per plant (totally 9), and for the other cultivars the number of HR lines varied from 8 in RO to 4 in AN (Table 1). For graphical presentation and statistical evaluation of PA data from different plant tissues and HRs (Fig. 1) only the cultivars DP, YS, LR and RO were considered, for which HRs from all three original plants were available.

Table 1
Levels of falcarinol (FaOH) and falcarindiol (FaDOH) in hairy root cultures and periderm samples obtained from seven carrot cultivars.

Cv.	Plant	FaOH Level [µg (g dry weight)$^{-1}$]					FaDOH Level [µg (g dry weight)$^{-1}$]				
		HR Line#1	*HR Line#2*	*HR Line#3*	*HR Mean*[1]	*Periderm*	*HR Line#1*	*HR Line#2*	*HR Line#3*	*HR Mean*[1]	*Periderm*
AN	*1*	2027	na[2]	na[2]	2027	934	2405	na[2]	na[2]	2405	1010
	2	1967	1558	1642	1722	442	4248	1521	1553	2440	1481
	3	na[2]	na[2]	na[2]	na[2]	793	na[2]	na[2]	na[2]	na[2]	1108
DP	*1*	2173	2082	4162	2805	551	2640	2436	2679	2585	1134
	2	3766	2358	1955	2693	576	1321	1905	1638	1621	972
	3	2862	3203	2101	2722	623	1522	2154	1522	1733	1596
YS	*1*	2047	3481	3374	2967	87	1407	1417	1407	1411	722
	2	1142	1219	1513	1291	51	826	696	1458	994	565
	3	2021	1692	2401	2038	493	2323	1931	2011	2088	1673
71	*1*	1877	1995	1580	1818	493	849	739	1255	948	720
	2	na[2]	na[2]	na[2]	na[2]	127	na[2]	na[2]	na[2]	na[2]	750
	3	2050	1642	1701	1798	287	678	721	582	660	900
LR	*1*	1994	1284	1822	1700	74	832	403	763	666	827
	2	1534	1155	2207	1632	27	984	1243	1105	1111	85
	3	1726	884	977	1196	38	2431	1010	1034	1491	416
RO	*1*	1683	1287	na[2]	1485	77	1164	1220	na[2]	1192	729
	2	2270	2306	1384	1987	193	807	660	885	784	649
	3	1841	1289	1448	1526	68	1301	1288	839	1143	574
SO	*1*	711	1682	1370	1254	70	848	940	1136	975	627
	2	1188	1133	1334	1219	128	1354	1234	946	1178	747
	3	na[2]	na[2]	na[2]	na[2]	512	na[2]	na[2]	na[2]	na[2]	1391

[1]mean level of HR lines developed from an individual plant, [2]not available

Rrhi strain ATCC 15834 proved to be highly effective for transformation of *D. carota*. This wild strain was used first in carrot transformation by Willmitzer et al. (1982) and has shown since then strong virulence in several plant studies. Among about 100 plant species, from which whole plants were regenerated from HRs carrying a wild type Ri plasmid, about twenty of them were transformed by the strain 15834 (Desmet et al. 2020). John et al. (2017) examined four different *Rrhi* strains, of which strain 15834 showed significantly higher transformation efficiency and was capable of

inducing HRs from different explants of *Achyranthes aspera*. Transformation of 'Indian ginseng' (*Withania somnifera*) was only successful with strain 15834 (Saravanakumar et al. 2012).

Two main PAs FaOH and FaDOH were quantified in carrot plant tissues and HRs. Among the five leaf and root tissues we detected the highest concentration of both compounds in the periderm samples, but there were large differences among the cultivars. Peridermal tissue of the purple cultivar DP accumulated approximately 600 µg/g DW (dry weight) FaOH, but in the orange cultivars LR and RO significantly lower FaOH levels of approximately 50 and 100 µg/g DW were registered, respectively. In DP the peridermal PA levels were significantly enhanced in comparison to the other plant samples (Fig. 1). However, due to the large variation of the PA data among the samples from individual plants most other differences were not significant. With regard to FaDOH, the purple cultivar DP also showed highest concentration in the periderm (1200 µg/g) whereas the peridermal FaDOH levels of the orange cultivars LR and RO were in the range of 450–650 µg/g DW (Fig. 1). The highest FaDOH content in the leaf samples was detected in DP with about 100 µg/g and in YS with 240 µg/g. PA accumulation patterns are influenced by numerous factors such as genotype, developmental stage, but also by abiotic and biotic stress factors. Besides, the PA distribution among the different plant organs and within the different root segments varies considerably (Dawid et al. 2015). Since the major aim of this study was not the analytical dissection of carrot plants but to determine the potential of PA production in HRs we analysed three selected individuals of each cultivar for HR induction and used leaf and root samples taken from each plant before *Rrhi* transformation as reference. Nevertheless, it was evident, that in all cultivars the periderm samples displayed the highest FaDOH levels. In addition, on an average of all cultivars, the concentration of FaDOH was 2 to 3 times higher as those of FaOH dependent of the plant tissue analyzed (data not shown). According to Czepa and Hofmann (2004) the most abundant PA in cultivated orange carrots is FaDOH, and the highest total PA levels were found in the periderm tissue (Baranska and Schulz 2005; Baranska et al. 2005). Busta et al. (2018) analysed tissue-specific accumulation of PAs in the orange cultivar 'Danvers' and also measured the highest total PA level in the peridermal tissues, with FaDOH as the dominating PA. With regard to FaOH, the purple cultivars DP (and AN, not shown) appeared to accumulate considerably higher contents in the periderm compared with the other tissue samples. It is generally recognized that the origin of the cultivated carrot was in Central Asia (Afghanistan), where purple-rooted wild carrots were domesticated and spread westward more than 1000 years ago (Simon et al. 2008). Orange carrots were not well documented until the 15th and 16th centuries in Europe (Stolarczyk and Janick 2011) which indicates a secondary domestication process. Purple, yellow, red, and white varieties persisted in Asia and the Middle East, and it can be assumed that current purple carrot cultivars are genetically closer to early domesticated purple carrots than to the modern orange varieties, which were selected for less bitterness. High PA concentrations were also observed in some wild relatives such as *D. carota* ssp. *maritimus, D. c.* ssp. *gummifer* and *D. c.* ssp. *commutatus* (Baranska et al. 2005).

Quantification of PAs in freeze-dried root samples of 51 individual HR lines revealed strong increases of the FaOH contents in all seven analysed cultivars ranging - on average of a single donor plant - from 1200 to 3000 µg/g DW in the HRs (Table 1). In case of the four cultivars DP, YS, RO and LR a significant increase of the FaOH level was observed in comparison to the periderm. For FaDOH there was also an increase in the HR lines (range 660 to 2600 µg/g DW) but compared with the periderm values the increase was less pronounced and not significant in the four fully analyzed cultivars (Table 1, Fig. 1). The HR line with the highest FaOH content was 'DP 1–3' with 4162 µg/g DW weight, which is about 7 times more than the content in the periderm of the original root. The HR line 'AN 2 – 1' contained with 4247 µg/g DW the highest FaDOH content of all lines (Table 1). Considering the moderate PA contents even in the periderm of the orange cultivars (LR, RO) there was an approximately 30-fold increase of the FaOH concentration in HRs of LR. This finding indicates, that the amount of FaOH may be considerably enhanced in HR cultures of cultivated carrots. The reason for this phenomenon is yet unknown but very probably caused by genetic factors. There is some evidence that fatty acid desaturases (FAD2) and their functionally divergent variants designated as acetylenases play the major role in the biosynthesis of C_{17}-PAs such as FaOH and FaDOH (Minto and Blacklock 2008; Busta et al. 2018; Jeon et al. 2020). However, it is widely unknown which members of the FAD2 gene family are controlling the functionally divergent genes

and which enzymes and genes are involved in the biosynthesis of FaDOH from its precursor FaOH. It might be possible, that orange cultivars were selected during the domestication process for genetic variants of genes involved in PA biosynthesis to avoid too much bitterness in the outer layer (periderm) of the root, and that this/these gene(s) are involved in the strong accumulation of FaOH in the HRs of orange carrot genotypes.

Our study suggests that the usage of field-grown carrot chemotypes, as for instance purple cultivars, might be a possibility for the commercial production of pharmaceutically relevant PAs. However, carrot HR cultures might be a suited alternative plant source, and biotechnological methods based on HRs such as bioreactor techniques seem to be a worth considering alternative. HR cultures, capable of synthesising a wide spectrum of phytochemicals, have been developed from numerous plant species (Malik 2017; Gutierrez-Valdes et al. 2020). However, only few reports are available about falcarinol-type PA production in HRs. FaOH was among the main components of oil samples from HR cultures of the Apiaceae species *Levisticum officinale* (Santos et al. 2005), and Washida et al. (2003) reported the production of FaOH and FaDOH in HRs of *Panax* hybrids. HRs of *Panax ginseng* were used to investigate the natural formation of FaOH and FaDOH by ^{13}C-labeling experiments (Knispel et al. 2013).

A major bottleneck in functional gene studies in non-model plants is often the lack of an efficient transformation protocol for recalcitrant genotypes or whole species. Since it is often easier to induce HRs using *R. rhizogenes* than to generate transformants using *A. tumefaciens*, HR-based functional gene studies might be an alternative (Guillon et al. 2006). In addition, if root-derived natural compounds are the target for approaches based on genome editing methods such as CRISPR/Cas9, HRs may be the method of choice because plant regeneration normally is not necessary. In this study we have shown that HR cultures of cultivated carrots may provide an efficient tool to assess the function of candidate genes involved in the biosynthesis of key PAs such as FaOH and FaDOH.

Declarations

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Declarations

The authors declare that they have no conflict of interest.

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Figures

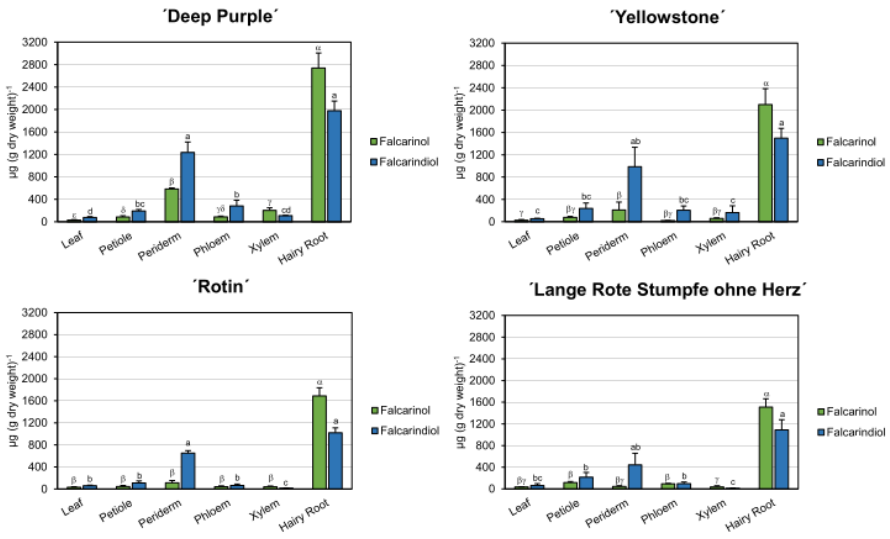

Figure 1. Falcarinol and falcarindiol levels in different leaf and root tissues and hairy roots. Bars represent means and error bars standard errors of mean (leaf, petiole, periderm, phloem, xylem: n = 3; hairy root: n = 9 for DP, YS and LR; n = 8 for RO). Significance analyses were performed after log-transformation using Tukey´s HSD test. Means with the same latin or greek letter are not significantly different ($P \geq 0.05$)

Figure 1

See image above for figure legend.