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Carnivory on demand: phosphorus deficiency induces glandular leaves in the African liana *Triphyophyllum peltatum*

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Summary

- *Triphyophyllum peltatum*, a rare tropical African liana, is unique in its facultative carnivory. The trigger for carnivory is yet unknown, mainly because the plant is difficult to propagate and cultivate. This study aimed at identifying the conditions that result in the formation of carnivorous leaves.
- *In vitro* shoots were subjected to abiotic stressors in general and deficiencies of the major nutrients nitrogen, potassium and phosphorus in particular, to trigger carnivorous leaves' development. Adventitious root formation was improved to allow verification of the trigger in glasshouse-grown plants.
- Among all the stressors tested, only under phosphorus deficiency, the formation of carnivorous leaves was observed. These glandular leaves fully resembled those found under natural growing conditions including the secretion of sticky liquid by mature capture organs. To generate plants for glasshouse experiments, a pulse of 55.4 μM α-naphthaleneacetic acid was essential to achieve 90% *in vitro* rooting. This plant material facilitated the confirmation of phosphorus starvation to be essential and sufficient for carnivory induction, also under *ex vitro* conditions.
- Having established the cultivation of *T. peltatum* and the induction of carnivory, future gene expression profiles from phosphorus starvation-induced leaves will provide important insight to the molecular mechanism of carnivory on demand.

Introduction

Carnivory, which for plants is defined as the ability to absorb nutrients from an animal prey captured by specific organs, has evolved several times (Givnish, 2015). Carnivorous plants belong to 20 genera in 12 families and five orders and comprise prominent examples such as Venus flytrap (*Dionaea muscipula*), sundews (*Drosera* sp.), bladderworts (*Utricularia* sp.), or butterworts (*Pinguicula* sp.) (Givnish, 2015). One of these plant species, *Triphyophyllum peltatum*, stands out due to the fact that carnivory is facultative, and the triggers of this switch in nutritional lifestyle are not understood up to now, mainly because the plant is difficult to cultivate.

Triphyophyllum peltatum (Hutch. et Dalz.) Airy Shaw is a unique plant species in terms of botanical and phytochemical properties (Airy Shaw, 1951; Bringmann *et al.*, 1998). The monophyletic genus *Triphyophyllum* belongs to the Dioncophyllaceae, which are closely related to the Ancistrocladaceae (Meimberg *et al.*, 2000). This endangered and rare species is native to the tropical moist broadleaf forests of tropical West Africa (Sierra Leone, Liberia, Ivory Coast) and colonises different altitudes of hillsides close to the Atlantic coast (Green *et al.*, 1979; Porembski & Barthlott, 2003). This habitat is characterised by an equatorial monsoon climate (Jones *et al.*, 2013), that is quite high temperatures (annual mean temperature: 28–29°C), relative air humidities of 70–75% and a disparate distribution of precipitation over the year, with a dry season from November to May with 3 months of < 10 mm rain per month (Green *et al.*, 1979). The plants are growing in shallow acidic soils of the Ferralsol soil group being strongly weathered and having low nutrient levels (Jones *et al.*, 2013).

Interest in *Triphyophyllum peltatum*, which is traditionally used in folk medicine (Porembski & Barthlott, 2003), is based on its rich production of pharmaceutically active secondary metabolites, namely acetogenic naphthoquinones (Bringmann *et al.*, 2016a), tetralones and structurally unique, axially chiral naphthylisoquinoline alkaloids (Bringmann *et al.*, 2000). Among these alkaloids, dioncopeltine A (François *et al.*, 1997), dioncophylline B (François *et al.*, 1999), dioncophylline C (François *et al.*, 1997) and habropetaline A (Bringmann *et al.*, 2003) have strong antiplasmodial activities (François *et al.*, 1997). Moreover, antitumoral and anti-MM (multiple myeloma) activities were shown i.a. for dioncoquinones A and B and for some of the alkaloids (Bringmann *et al.*, 2008).

As indicated by the genus name *Triphyophyllum*, the plant produces three types of leaves: Juvenile shoots develop either

oblanceolate or glandular leaves, whereas adult liana shoots form leaves with hooks supporting the climbing growth of the mature shoots (Green *et al.*, 1979). The oblanceolate and the liana leaves represent the autotroph type. The leaves of the carnivore-ecomorph type are glandular leaves and carry stalked and sessile glands, which secrete a clear, viscid fluid (Marburger, 1979) containing proteases, peroxidases, esterases and acid phosphatases (Green *et al.*, 1979). Many Coleoptera and other insects stick to the glands suggesting carnivory (Airy Shaw, 1951; Porembski & Barthlott, 2003). Bringmann *et al.* (2001) proved the uptake of ^{13}C-labelled alanine by the glandular leaves, which was transported and could be detected in the leaves. The carnivorous leaves were observed in natural stands to be produced in highest numbers – but not exclusively – from March to June, shortly before the onset of the rainy season (Green *et al.*, 1979). Seedlings of different ages form the glandular leaves and transitional leaves with basal parts having a lamina and only the tips carrying glands were reported (Green *et al.*, 1979).

The rare plant is difficult to cultivate, although progress in propagation has been reached by Bringmann & Rischer (2001) and Rembold *et al.* (2010), who described an *in vitro* multiplication protocol for *T. peltatum*. Limiting in this tissue culture approach was, however, the difficulties in rooting with a maximum of 27% rooting percentage. Under *in vitro* conditions, Bringmann & Rischer (2001) observed carnivorous glandular leaves only in exceptional cases on adventitious shoots regenerating from callus. Nevertheless, the possibility to establish *in vitro* cell (Bringmann *et al.*, 2016b) and root cultures (Bringmann *et al.*, 2016a) may help to preserve germplasm *ex situ*.

Although already Green *et al.* (1979) and Marburger (1979) suggested that carnivory in this tropical liana is associated with nutrient deficiency, up to now, the difficulties in propagation and cultivation of *T. peltatum* have impeded the identification of the trigger for the induction of carnivory. The objective of this study was to identify this trigger under controlled *in vitro* culture and *ex vitro* glasshouse conditions. We here report on the establishment of *in vitro* rooting and carnivorous leaf development. The obtained knowledge regarding carnivory induction provides the basis for analysing the molecular ground of *T. peltatum*'s carnivory on demand.

Materials and Methods

Plant material and *in vitro* culture conditions

In vitro shoot cultures of one *Triphyophyllum peltatum* (Hutch. et Dalz.) Airy Shaw genotype that had been established from seeds and maintained according to Bringmann & Rischer (2001) were available from previous work (Bringmann *et al.*, 1991; Rembold *et al.*, 2010). Several media were tested to optimise growth and propagation resulting in basal medium 46.15 (Tables 1, 2), which gave rise to the best shoot quality. The medium contained modified MS (Murashige & Skoog, 1962) macro-elements (half strength), modified MS micro-elements, B5 (Gamborg *et al.*, 1968) vitamins, 30 g l^{-1} sucrose and 4 g l^{-1} Gelrite (Duchefa, Haarlem, the Netherlands) at pH 5.8. Axillary shoot propagation was achieved by subculturing the tips and the bases of the shoots and collecting emerging side shoots every 2 months. All shoot cultures were incubated in 250-ml polypropylene vessels filled with 70-ml culture medium at 24 ± 2°C and under a 16-h photoperiod at 35–

Table 1 Composition of the different *in vitro* culture media.

	Medium			
	46.15 (basal medium)	46.27 (N deficiency)	46.28 (K deficiency)	46.29 (P deficiency)
Macro-elements (mM)				
NH_4NO_3	10.3	–	10.3	10.3
KNO_3	9.4	–	–	10.0
$MgSO_4$	1.5	1.5	1.5	1.5
$CaCl_2 \times 2\ H_2O$	2.0	2.0	2.0	3.0
KH_2PO_4	0.6	1.3	–	–
K_2SO_4	–	4.4	–	–
$Ca(NO_3)_2$	–	–	4.7	–
NaH_2PO_4	–	–	0.6	–
N	30.0	0	30.0	30.6
K	10.0	10.1	0	10.0
P	0.6	1.3	0.6	0
Estimated P from Gelrite	0.14	0.14	0.14	0.14
Micro-elements (μM)				
FeNaEDTA	150.0	150.0	150.0	150.0
H_3BO_3	100.3	100.3	100.3	100.3
$MnSO_4 \times H_2O$	100.0	100.0	100.0	100.0
$ZnSO_4 \times 7\ H_2O$	29.9	29.9	29.9	29.9
KI	5.0	5.0	5.0	5.0
$Na_2MoO_4 \times 2\ H_2O$	1.0	1.0	1.0	1.0
$CuSO_4 \times 5\ H_2O$	0.1	0.1	0.1	0.1
$CoCl_2 \times 6\ H_2O$	0.1	0.1	0.1	0.1
Vitamins and other organic compounds (μM)				
Myo-inositol	554.9	554.9	554.9	554.9
Thiamine HCl	29.7	29.7	29.7	29.7
Nicotinic acid	8.1	8.1	8.1	8.1
Pyridoxine HCl	4.9	4.9	4.9	4.9
Sucrose (mM)	87.6	87.6	87.6	87.6
Gelrite (% w/v)	0.4	0.4	0.4	0.4

Grey cells highlight the deviations from the basal medium. Dark grey cells indicate an estimation of the phosphorus load of Gelrite (batch A). Plant growth regulator concentrations are given in Table 2.

Table 2 Plant growth regulator combinations and concentrations tested in the different *in vitro* culture media.

		Concentration (μM)				
Medium	Purpose	Kin	BA	IAA	IBA	NAA
46.15	Propagation	2.3	1.1	5.7	–	–
46.12	Root induction	–	–	–	24.6	–
46.13	Root expression	–	–	–	–	–
46.16	Root induction	–	–	–	–	26.9
46.17	Root induction				24.6	26.9
46.19	Root induction				55.4	
46.20	Root induction					55.4
46.21	Root induction					110.8
46.26, 46.27, 46.28	Induction of carnivorous leaves	2.3	1.1	5.7	–	–

BA, 6-benzylaminopurine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kin, kinetin; NAA, α-naphthaleneacetic acid.

40 µmol m^{-2} s^{-1} (PPFD-PAR) provided by cool daylight fluorescent tubes (Philips Master TL-D 58 W/865; Signify, Hamburg, Germany, or Osram Lumilux 58 W/865, München, Germany).

In vitro rooting experiments

A total of eight rooting experiments were carried out submitting *in vitro* shoots to different root induction (1 wk) and root expression (6 wk) treatments (Table 3). Root induction media (Tables 1, 2) contained the auxins IBA and NAA in different concentrations and combinations, whereas expression media were free of plant growth regulators. For root expression, either autoclaved Seramis (https://www.seramis.com: granules of clay and lava, 85 ml + 30 ml sterile deionised water), medium 46.13 (Tables 1, 2), water agar (0.8% (w/v) Plant agar; Duchefa, in tap water) or water agar plus 2 g l^{-1} activated charcoal were used. Culture conditions were the same as described above for shoot propagation. At the end of the experiments, the explants were carefully washed to count the adventitious roots and to measure the longest root.

Carnivory induction experiments *in vitro*

In a first set of experiments, abiotic stress including high-light conditions, heat and cooler temperatures, as well as stress hormones, such as ethylene or jasmonic acid and gibberellic acid were applied (Supporting Information Table S1).

The next experiments focussed on nutrient deficiencies. *In vitro* culture media were designed (Table 1), which were deficient in either nitrogen (N: 46.27), potassium (K: 46.28) or phosphorus (P: 46.29). The macro-element composition was adjusted in a way that the companion ions of the respective salts were offered in a comparable concentration as in the basal medium. Sixty shoots each (six vessels with five shoots in two experimental repetitions = exp. A and B) were transferred from the basal medium 46.15 to the four media 46.15, 46.27, 46.28 and 46.29 and cultured under the conditions specified above. Every 2–3 months, the shoots were subcultured. They were observed weekly for the formation of glandular leaves for *c.* 6 months. To study P deficiency-induced carnivory in more detail, further experiments (exp. C, D) comparing media 46.15 and 46.29 were carried out using the same settings, except for the starting material, which had not been subcultured for 5 months. In addition, the shoots were transferred to fresh media every 4 wk instead of every 2 months.

In an additional experiment, five liquid media were compared differing in their KH_2PO_4 concentration (0–0.63 mM, Table S2) to avoid the gelling agent Gelrite to provide phosphorus to the shoots. The control medium corresponded to medium 46.15 (Tables 1, 2 basal medium) without Gelrite, but the concentrations of the plant hormones were reduced to one-fifth due to their higher availability in the liquid media. The shoots were fixed in openings of a nylon mesh, which had been melted onto polypropylene rings to position the mesh at a height of 1.5 cm. These holdings were placed in 500-ml plastic culture vessels and filled with 60 ml of the respective medium. Four shoots were cultured in one vessel, and six replicate vessels were used. The medium was exchanged once after 7 wk and filled up to the mesh height after 15 wk. The number of shoots forming glandular leaves and the number of glandular leaves per shoot (considering all shoots) were recorded weekly until the final evaluation after 21 wk.

Table 3 Overview of the *in vitro* rooting experiments.

Experiment no.	Root induction media	Root expression media	Replicate no. (vessels with five shoots each) per treatment
1	46.12; 46.16; 46.17	Seramis; WA	4
2	46.17; 46.19; 46.20	WA	6
3	46.17	Seramis; WA; 46.13	4
4	46.17; 46.19; 46.20	WA	6
5	46.17	Seramis; WA; 46.13	4
6	46.17; 46.20; 46.21	WA + AC	4
7	46.17; 46.20; 46.21	WA + AC	4
8	46.17; 46.20; 46.21	WA + AC	4

Media composition is given in Tables 1 and 2; Seramis = (85 ml Seramis + 30 ml sterile deionised water); WA = water agar (0.8% (w/v) Plant agar; Duchefa); WA + AC = water agar plus 2 g l^{-1} activated charcoal.

Nutrient analyses

After 6 months of *in vitro* cultivation on either control (46.15), N, K or P deficiency (45.27, 45.28, 45.29), whole shoots were pooled to reveal a minimum of 250 mg dry mass (DM) after drying at 70°C for 72 h. The samples were ground to a fine powder in 2-ml reaction tubes with 4-mm steel beads in a mixer mill (MM400; Retsch, Haan, Germany) at 30 Hz for 2 min. Following the procedure by Gericke & Kurmies (1952), 200 mg pulverised sample was reduced to ash at 480°C overnight before being dissolved in 5 ml 21.7% w/v nitric acid. Thereafter, the solution was transferred quantitatively with deionised water into a 50-ml volumetric flask and brought to volume. After filtration with blue band filter (2–3 µm), contents of B, Ca, Cd, Cu, Fe, K, Mg, Mn, Mo, and Zn were analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES; Varian 725-ES, Varian Inc., Palo Alto, CA, USA).

Phosphorus was measured on a photometer (Cary50 Scan; Varian Inc.) after dyeing based on the ammonium vanadate molybdate method of Gericke & Kurmies (1952).

For determining total carbon and nitrogen contents, 5–7 mg of the pulverised DM was used. The analyses were performed by dry combustion on a Vario EL III elemental analyser (Elementar Analysensysteme, Hanau, Germany). Acetanilide was used as a calibration standard (Merck KGaA, Darmstadt, Germany) with 71.1% C and 5.95% N.

Carnivory induction experiment *ex vitro*

For acclimatisation, *in vitro* rooted plantlets were carefully washed and planted in 7-cm pots filled with a loamy subsoil

(Luvisol from loess) taken at a depth of 40–130 cm from the Deister Forest close to Hanover, Germany (collected by and characterised in Dechassa *et al.*, 2003). The soil texture was dominated by silt (12% clay, 84% silt, 5% sand), and it was chosen due its low content of phosphorus of 16 mg kg^{-1} (Dechassa *et al.*, 2003). The plants were cultivated under a foil tent in a glasshouse for 3 wk (8 August 2022–29 August 2022) at 26°C (set temperature) and 16 h of additional light provided by SON-T Philips Master Agro 400 W lamps (Hamburg, Germany) from October to March. Under the closed foil tent, no plant protection treatments were necessary. Starting 3 wk after acclimatisation, 39 plants were fertilised by daily spraying the leaves with Wuxal Super containing 2.8 mM Pi (Pi: orthophosphate) (Aglukon Spezialduenger GmbH & Co. KG, Düsseldorf, Germany; liquid foliar fertiliser, N–P–K (% w/w): 8–8–6, https://www.mywuxal.com/en/product/super), whereas 40 plants in a second foil tent were sprayed daily with a fertiliser solution containing 0.1 mM Pi to induce phosphorus deficiency. This solution was prepared freshly every week by mixing 964 ml of 0.1% Wuxal Combi Mg (Aglukon Spezialduenger GmbH & Co. KG; foliar fertiliser suspension, N–K (% w/w): 20–15, https://www.mywuxal.com/en/product/combi-mg) and 36 ml of 0.2% Wuxal Super. Using this mixture, the macro- and micro-elements other than phosphorus were applied in comparable concentrations. The plants were observed weekly for the formation of glandular leaves.

Statistical analyses

Data from the rooting experiments, that is percentage of rooted shoots per vessel, number of roots per rooted shoot and length of the longest root and the nutrient concentration data, were analysed in a linear model using the R program (v.4.0.5, R Development Core Team, 2021) under RSTUDIO. The following packages were employed: TIDYVERSE (Wickham *et al.*, 2019), DPLYR (Wickham *et al.*, 2021) and EMMEANS (Lenth, 2022). An ANOVA with the treatment as a factor followed by pairwise comparisons of means with Tukey's test ($P < 0.05$) was calculated for the root number and length data after log transformation as well as nutrient concentrations. For rooting percentage, the nonparametric Kruskal–Wallis test and pairwise comparisons using Wilcoxon's rank sum tests ($P < 0.05$) were chosen. In the *in vitro* experiment employing liquid medium for glandular leaf induction, the residuals were not normally distributed due to many zero values. Therefore, the data on average numbers of glandular leaves (based on all living shoots of the respective treatment) were submitted to the nonparametric Kruskal–Wallis test followed by pairwise comparisons using Wilcoxon's rank sum tests ($P < 0.05$).

Results

To identify the trigger for induction of carnivory, we conducted a series of experiments under varying *in vitro* conditions. These *in vitro* conditions, and the mixotrophic growth in particular, are artificial, but they allow the controlled investigation of nutrient effects excluding disturbing microbial activities for example. To make the step to the glasshouse (*ex vitro*), we first had to establish an efficient *in vitro* rooting system.

NAA pulse is the key for *in vitro* rooting

In most *in vitro* propagation protocols, adventitious root formation is the final phase before the rooted plantlets are transferred to *ex vitro* conditions. Based on eight rooting experiments, media were optimised for the two subsequent phases in adventitious root formation, induction and expression (Druege *et al.*, 2016). Given that the induction phase requires auxin, high concentrations of 24.6–110.8 mM of the two strong synthetic auxins IBA and NAA were applied in this first phase. Only few shoots (≤ 3%) formed roots if only IBA was used (Fig. 1), whereas 26.9 µM NAA alone or in combination with 24.6 µM IBA resulted in significantly higher rooting percentages of 40–77%. The highest rooting percentage of 90% was reached when 55.4 µM NAA were applied for induction, followed by expression on water agar with activated charcoal (WA + AC) (Fig. 1). Regarding the expression media, nutrient medium with mineral salts, sucrose and vitamins (medium 46.13, Tables 1, 2) resulted in lower percentages of rooted shoots than Seramis moisturised with water, water agar (WA) and WA + AC.

At the end of the 7-wk rooting period, the shoots had formed roots with long blackish root hairs (Fig. 2a–d). Most treatments resulted in three to five roots per rooted shoot (Fig. S1). Again, 55.4 µM NAA for induction and WA + AC for expression was the best combination leading to 5.8 roots per rooted shoot (Fig. S1). Neither the induction nor the expression medium significantly affected root length (varying from 2.5 to 8.2 mm) (Fig. S2).

P deficiency gives rise to carnivorous leaves under *in vitro* conditions

Under the standard conditions during *in vitro* multiplication and rooting, carnivorous leaves were not observed. This situation did not change when testing several abiotic stress factors and stress hormones (Table S1).

Green *et al.* (1979) considered the formation of carnivorous leaves to be a reaction to nutrient deficiency – possibly a lack of potassium, whereas Marburger (1979) stated that the nutrient-poor soils of a *T. peltatum* habitat in Sierra Leone result in an ecological pressure leading to the induction of carnivory to obtain nitrogen and Ellison (2006) predicted *T. peltatum* to be co-limited in growth by N and P. To place nutrients limitation experiments on solid grounds, we took advantage of our *in vitro* culture system. Essentially, we tested for the three major nutrient elements, that is nitrogen, potassium and phosphorus. Nitrogen deficiency caused chlorotic and small leaves on the shoots after 6-month culture on medium 46.27 (Table 4). This phenotype was in line with the significantly lower N concentration of 8.4 mg g^{-1} DM (dry mass) in these shoots compared with shoots on control medium 46.15 (20.7 mg g^{-1} DM) (Table 5). However, the depletion of N in the shoots did not induce any glandular leaves. Potassium deficiency was also successfully induced as indicated

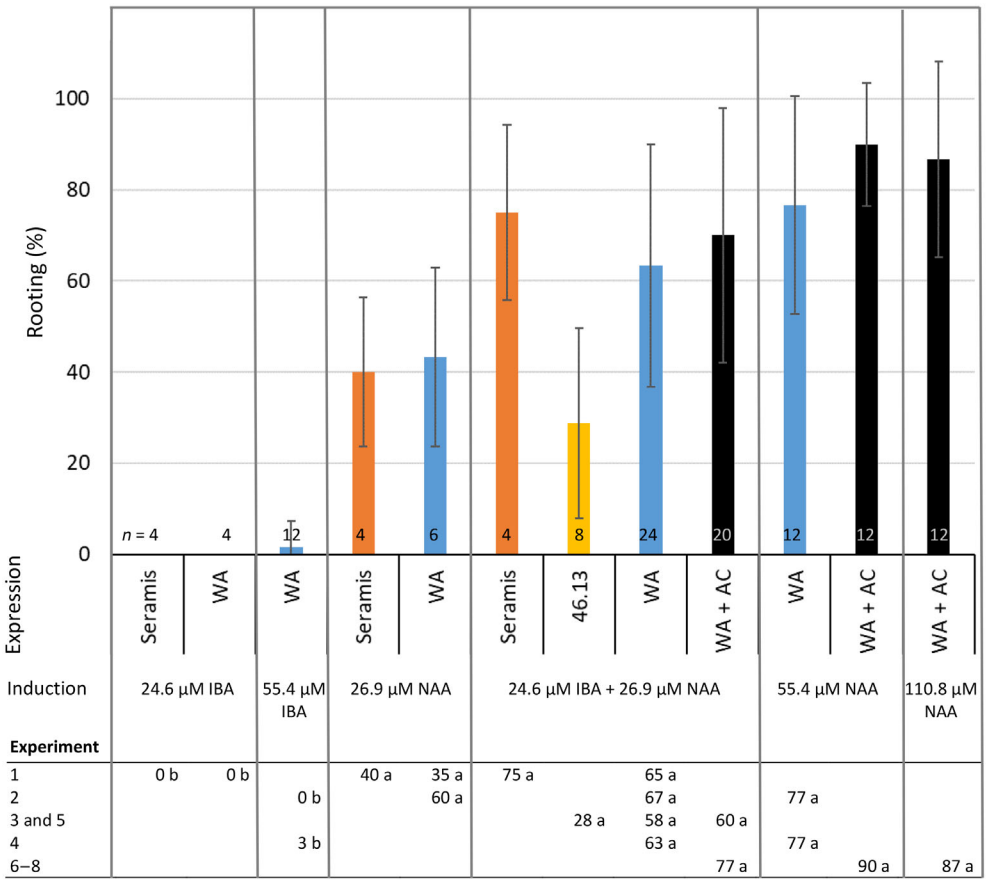

Fig. 1 Rooting percentage of *Triphyophyllum peltatum in vitro* shoots in eight rooting experiments (listed in Table 3). Data are means ± SD of *n* replicates (vessels with five shoots each). Treatments within experiments were compared by Wilcoxon's rank sum tests at $P < 0.05$.

by the nutrient concentrations in the shoot dry mass (Table 5), but resulted neither in pronounced visual symptoms nor in the development of carnivorous leaves (Table 4). Shoots on medium 46.28 (K deficiency) contained Ca concentrations, which were twice as high as in shoots of the other treatments (Table 5).

Shoots on P deficiency medium (46.29, Table 1) were proven to have significantly lower P concentrations of 0.59 mg g^{-1} DM compared to 1.6 mg g^{-1} DM for shoots from control medium (Table 5). Under P deficiency, the leaves were narrower and older ones turned brown. But, most importantly, 67% of the shoots had developed at least one glandular leaf (Table 4). Shoots with glandular leaves tended to have lower P concentrations (0.60 mg g^{-1} DM) than shoots from the same vessels, which had not developed glandular leaves (0.71 mg g^{-1} DM) ($n = 3$).

Carnivorous leaves grown *in vitro* under P starvation develop secreting glands

The development of the glandular leaves started with a characteristic enrolling of the leaf tips covered with green glands (Fig. 3a, b). Within a few days, glands turned red and developed into sessile and erect glandular structures (Fig. 3c,d). About 1–2 wk later, a sticky liquid was found to be secreted starting at the glands at the base. This secretion was only observed at the erect glands (Fig. 3e,f). The longevity of this last stage of glandular leaf development was *c.* 1–2 months. On most shoots, the first carnivorous leaves were transitional leaves with a laminar leaf base (Fig. S3). If shoots with glandular leaves were transferred back to control medium, the newly formed leaves were all of the normal oblanceolate type without traps (Fig. S3). Overall, the glandular leaves perfectly resembled those described for *ex vitro* plants at their natural stands (Marburger, 1979).

For the quantification of glandular leaf formation over time, additional experiments (exp. C and D, see the Materials and Methods section) were undertaken, comparing shoots cultured on control medium 46.15 with shoots on P deficiency medium 46.29. In these experiments, the induction of carnivorous leaves was confirmed and, as in experiments A and B, about two-thirds of all shoots developed glandular leaves after 24–28 wk (Fig. S4). However, in experiments C and D, carnivorous leaves were observed earlier and in higher numbers of 1.7 and 3.1 per shoot, respectively (Fig. S4). The major difference to experiments A and B was that shoots had not been subcultured for 5 months before starting experiments C and D.

Additional experiments followed in which not a single glandular leaf was observed even after 7.5 months of culture on P deficiency medium 46.29. Since the only considerable P source in the medium could have been in the gelling agent Gelrite and since a new batch (B) had been opened when these experiments were started, four different batches of Gelrite (all from Duchefa)

Fig. 2 Formation of adventitious roots on *Triphyophyllum peltatum in vitro* shoots. (a) Plantlets after 1 and 6 wk of culture on root induction (46.17, Table 2) and expression medium (WA + AC), respectively. (b) Rooted plantlet after 1 and 6 wk of culture on root induction (46.17) and expression medium (WA), respectively. (c) Rooted plantlet after 1 and 6 wk of culture on root induction (46.20 Table 2) and expression medium (WA + AC), respectively (Bar, 1 cm). (d) Roots of the plantlet shown in (c) in water (Bar, 200 µm).

were analysed for P. Indeed, the P concentrations differed significantly (by a factor of 1.5) explaining the observations (Notes S1) and emphasising the need to consider contribution of nutrients in gelling agents in deficiency experiments *in vitro*.

In order to circumvent difficulties with nutrient loads of gelling agents, an experiment with increasing Pi concentrations in liquid medium was performed. Again on the control medium containing 0.63 mM Pi, no glandular leaves were formed. With decreasing Pi supply, an earlier induction of carnivory was observed with shoots cultured without any Pi in the medium starting to develop glandular leaves after 10 wk (Fig. 4). Variants supplemented with 0.05 and 0.1 mM Pi followed in Week 13 and shoots on 0.2 mM Pi in Week 17. Over time, the number of glandular leaves increased in all variants except for the control, resulting in more than three glandular leaves per shoot in media containing 0 mM Pi and 0.1 mM Pi (Fig. 4) and in 96–100% shoots with glandular leaves (Fig. S5). Typical P deficiency symptoms were observed, such as browning of the older leaves starting from the leaf tips and later dying of older leaves, the intensity and time point being clearly dependent on the Pi concentration of the medium (Fig. S6).

Carnivory is also induced *ex vitro* under P starvation

The developed *in vitro* rooting protocol allowed us to translate the findings from the *in vitro* experiments to glasshouse conditions. In a first approach, 10 young plants were fertilised without Pi and compared to those fertilised with 2.8 mM Pi. Under P deficiency, the leaves of nine plantlets increasingly turned brown to black and were shed until the plants died after 3 months. The surviving plant, however, formed a glandular leaf (Fig. S7) and had P concentrations of 0.91 mg g^{-1} DM and 0.55 mg g^{-1} DM in the stem and the leaves, respectively. Since the complete omission of P from the foliar fertiliser obviously did not allow the young plants to acclimatise and survive this treatment, for the *ex vitro* induction experiment, the plants in the Pi deficiency variant received fertiliser solution containing 0.1 mM Pi by foliar application. The plantlets developed well and did not show P deficiency symptoms in the 0.1 mM Pi variant. Strikingly, after 11 wk, the first carnivorous leaf was noticed in this variant (Fig. S8). The number of glandular leaves increased over time and after 24 wk 13% of the plants had developed at least one. Mostly, smaller plants started to induce carnivory until the final evaluation, so the experiment likely needed to be continued to allow for Pi depletion also in the bigger plants. By contrast, none of the plants fertilised with 2.8 mM Pi developed carnivorous leaves. Thus, P deficiency triggering carnivory was confirmed under glasshouse conditions.

Discussion

An optimised *in vitro* rooting protocol guarantees material for carnivory induction under glasshouse conditions

Rooting was described to be the major bottleneck in *in vitro* propagation (Bringmann & Rischer, 2001). We could

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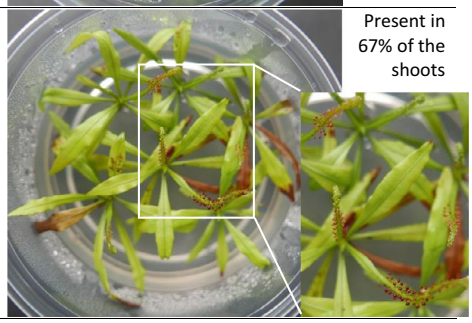
Table 4 Nutrient deficiency variants tested to induce glandular leaves *in vitro*.

Treatment	No. of shoots	Duration of exp. (wk)	Observations		Glandular leaves
Control: medium 46.15	120	24–28	Green and healthy leaves Photo taken after 24 wk		None
N deficiency: medium 46.27	60	28	Chlorotic leaves, brown leaf tips, poor growth Photo taken after 28 wk		None
K deficiency: medium 46.28	60	24	Shoots elongated Photo taken after 24 wk		None
P deficiency: medium 46.29	120	28	Reduced growth, narrow leaves with brown spots and margins Photo taken after 28 wk		Present in 67% of the shoots

overcome this limitation and reproducibly achieved 90% of rooting by a two-step protocol including 1 wk of induction on an auxin-containing medium followed by 6 wk of root growth on plant growth regulator-free media (Fig. 1). This regime addressed the known different hormonal requirements during the different phases of adventitious root formation, which are defined as dedifferentiation (De Klerk *et al.*, 1999) and induction (sometimes considered as one phase, Druege *et al.*, 2016), initiation and expression (Bellini *et al.*, 2014; Druege *et al.*, 2019). During the first phase of induction (including dedifferentiation), an epigenetic reprogramming takes place, which is mainly triggered by auxin (Druege *et al.*, 2019). A very clear superiority of NAA in adventitious root induction was observed, whereas its concentration had only a minor effect in the range tested (Figs 1, S1). NAA is a strong synthetic auxin that is commonly used for difficult-to-root species. It expressed the highest root inducing activity when compared to IAA and IBA on *Arabidopsis thaliana* floral stem segments (Verstraeten *et al.*, 2013). It is assumed that different effects of auxins in different plant species are due to

Table 5 Nutrient concentrations in *Triphyophyllum peltatum in vitro* shoots cultivated for 6 months on medium 46.15 (control), 46.27 (N deficiency), 46.28 (K deficiency), or 46.29 (P deficiency) with subculturing every 2 months.

	Medium			
Mineral element	46.15	46.27 (N deficiency)	46.28 (K deficiency)	46.29 (P deficiency)
N (mg g^{-1} DM)	20.7 ± 1.1b	8.4 ± 0.1a	20.6 ± 0.6b	25.0 ± 2.3c
K (mg g^{-1} DM)	15.0 ± 2.1b	15.4 ± 1.5b	9.2 ± 1.1a	20.0 ± 1.5c
P (mg g^{-1} DM)	1.57 ± 0.30b	1.61 ± 0.09b	1.95 ± 0.25b	0.59 ± 0.05a
Ca (mg g^{-1} DM)	2.40 ± 0.60a	2.06 ± 0.08a	4.08 ± 0.55b	2.01 ± 0.23a
Fe (mg g^{-1} DM)	0.055 ± 0.009b	0.033 ± 0.004a	0.043 ± 0.003ab	0.128 ± 0.004c
% C	48.8 ± 0.9a	48.5 ± 0.3a	50.3 ± 0.4b	49.5 ± 0.4ab

Given are means and SDs of $n = 3$ replicates. Values indicated by the same letter within one row are not significantly different according to Tukey's test ($P < 0.05$). The intensity of the red colour increases with the concentration within one row (= element). DM, dry mass.

different binding affinities to auxin receptors and/or differences in auxin signalling (Pacurar *et al.*, 2014).

After the cellular reprogramming during the induction phase, the next phases of initiation and expression do not require auxin any longer; on the contrary, auxins may even have negative effects if present in this phase (De Klerk *et al.*, 1999). Thus, we employed auxin-free media in the expression phase. Among them, water solidified with agar or applied in Seramis granules better supported root development and growth than a nutrient medium (46.13, Fig. 1). Obviously, the lack of nutrients stimulated root outgrowth. However, because several nutrients were demonstrated to have promoting effects on adventitious root formation (reviewed in Druege *et al.*, 2019), it is possible that root outgrowth can be further stimulated by testing other optimised expression media, starting with the omission of sucrose. Seramis granules were included in the experiments, because they can be assumed to provide a better oxygen supply to the emerging roots. Seramis did not result in significant improvements regarding rooting percentage or root number, but in slightly longer roots (Figs 1, S1, S2). Activated charcoal slightly, but not significantly increased the percentage of rooted shoots and the root number (Figs 1, S1). Its effects can be explained by providing dark conditions to the cutting base and by its high adsorption capacity for phenolics, for example (Pan & van Staden, 1998).

In summary, the problems during *in vitro* rooting in *T. peltatum* were solved by the developed protocol, using a 1-wk 55.4 µM NAA pulse, followed by WA + AC for expression. Thus, *in vitro* propagated plantlets became available for the glasshouse experiments to induce carnivory.

Induction of carnivory by P deficiency

In its natural habitat, *T. peltatum* forms carnivorous leaves throughout the year, but with a maximum at the end of the dry season (Green *et al.*, 1979). The carnivorous phase, however, is not an obligate, but a facultative prerequisite for the phase change into the mature liana and finally flowering (Bringmann *et al.*, 2002). Also, axillary shoots formed after pruning mature plants may develop carnivorous leaves (Green *et al.*, 1979). These observations exclude developmental phase changes to be causal for the switch to carnivory and make nutrient limitation or abiotic stress more likely triggers for carnivory induction.

Phenotypic plasticity is commonplace, and theories predict that organisms should frequently evolve mechanisms to detect and respond to environmental cues such as limitation of a nutrient source. Here, we tested this prediction in *T. peltatum*. The tropical liana grows either leaves of the autotroph- or carnivore-ecomorph type. Under culture conditions tested until today, the formation of carnivorous leaves was not successful. We employed *in vitro* culture techniques to find the trigger giving rise to carnivory, and used a strategy based on abiotic stresses and nutrient diets. We found that in response to P deprivation and in turn a drop in shoot P concentration the switch in leaf phenotype from autotroph- to carnivore-ecomorph occurred. P deficiency was sufficient to induce glandular leaves, although it cannot be excluded that other external stimuli may modify this process. A similar behaviour was observed in heterophyllous *Cephalotus follicularis*, where the change in ambient temperature caused the switch between carnivory-dominated pitcher leaves vs photosynthesis-dominated flat leaves (Fukushima *et al.*, 2017).

The digestion of prey will provide the limiting nutrient P to *T. peltatum*. Likewise, Chandler & Andersson (1976) showed P concentrations in *Drosera* plants that had been cultivated in low-P conditions, to increase from 0.63 to 0.92 mg g^{-1} DM after feeding *Drosophila*. We observed *T. peltatum* to form carnivorous leaves at 0.6 mg P g^{-1} DM or less.

Phosphorus (P) is one of the most essential nutrients for the adequate growth and development of plants. It is an integral part of nucleic acids, energy metabolism intermediates and membrane phospholipids (White & Hammond, 2008) and is found in concentrations of 4–15 mg g^{-1} DM in well-nourished land plants (Broadley *et al.*, 2004), whereas carnivorous plants mostly contain < 1 mg P g^{-1} DM (Ellison, 2006). Plants take up P only in the inorganic form of orthophosphate (Pi), which has a rather low solubility and thus low mobility in the soil solution (White & Hammond, 2008). This fact could explain the peak in glandular leaf formation at the end of the dry season: The P availability is low anyway, in the marginal acidic Ferrasol soils of the natural habitat due to strong weathering, and P immobilisation at low

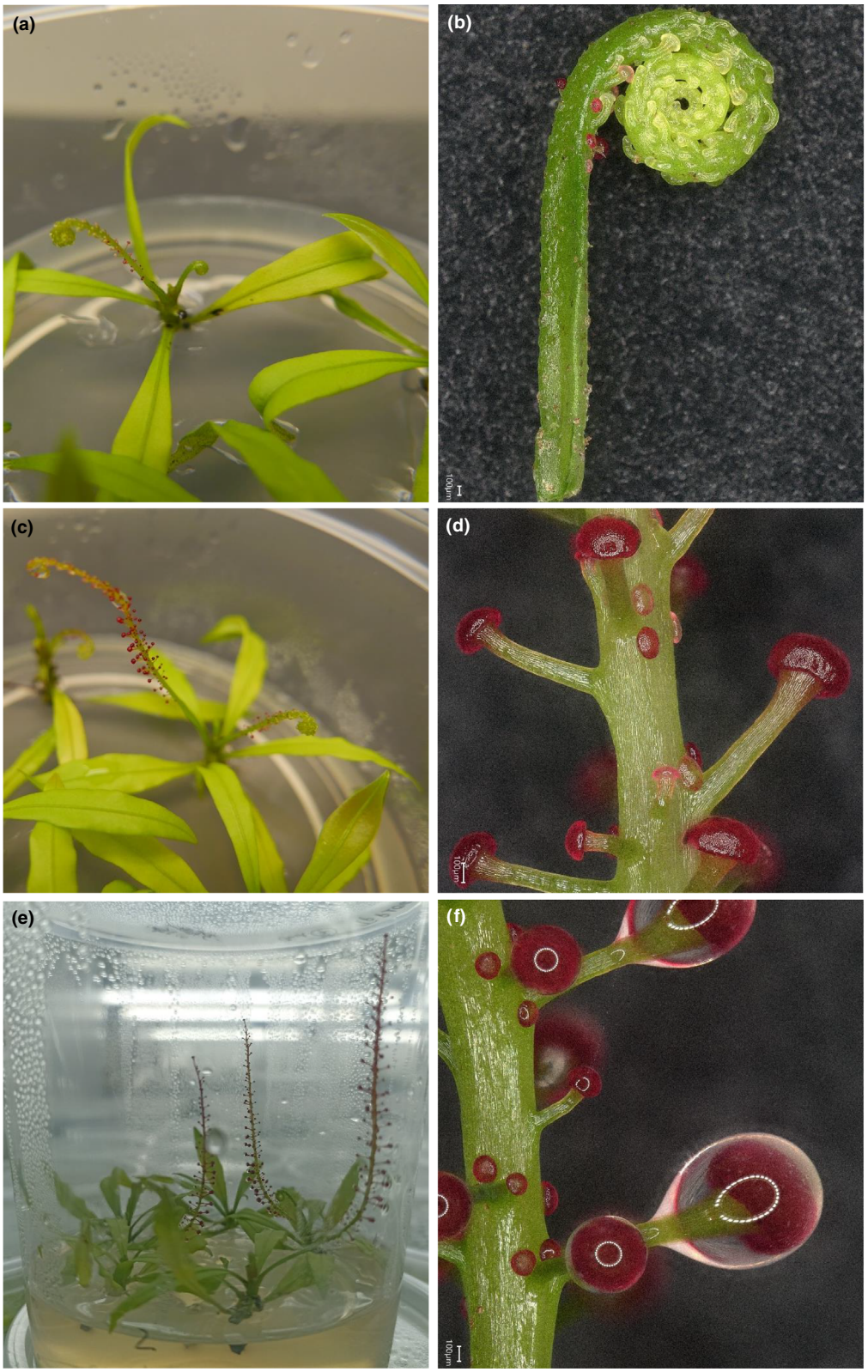


Fig. 3 Developmental stages of glandular leaves. (a, b) Early circinate stage, (c, d) erect stage with sessile and stalked glands, (e, f) late stage with glands carrying secretion droplets. (b, d, f) Stacked photographs taken under a digital microscope (Keyence VHX-7000).

pH to Fe and Al oxides (Jones *et al.*, 2013). But at the end of the dry season, the Pi concentrations in the soil solution can be expected to be strongly depleted. Plant-available Pi at the natural stands most likely is also strongly influenced by the growth cycles of the broadleaf trees in terms of Pi uptake but also litter fall and its decomposition.

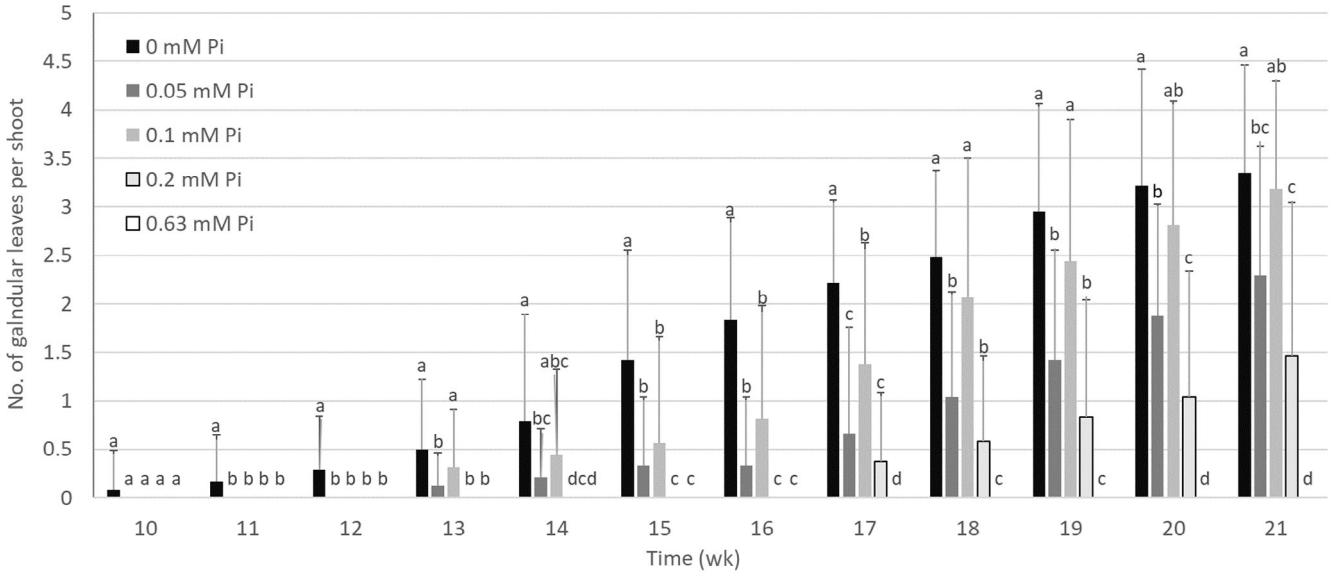


Fig. 4 Glandular leaf formation *in vitro* over time in liquid medium with different Pi concentrations. Given are means ± SD of *n* shoots. ($n = 24$ for variants 0 mM Pi, 0.05 mM Pi and 0.2 mM Pi; due to contaminations $n = 16$ for variants 0.1 mM Pi and 0.63 mM Pi). Values indicated by the same letter within one time point are not significantly different according to Wilcoxon's test ($P < 0.05$).

Comparison with the reaction to P starvation in noncarnivorous plants

Plants have evolved physiologically, biochemically and morphologically to cope with Pi starvation through investment into the root system, modification of the root system architecture, rhizosphere acidification and exudation of phosphatases, higher Pi uptake efficiency, remobilisation of the internal Pi (from older leaves) and establishing symbioses with mycorrhizal fungi (White & Hammond, 2008). Like other plant species, *Arabidopsis thaliana* roots, under Pi deficiency conditions, undergo striking morphological changes with mainly a reduction in primary root length while increasing lateral root length as well as root hair length and density (Bouain *et al.*, 2016). These major changes in root morphology and architecture seem to be initiated by a sensing mechanism in the root tip (Ticconi *et al.*, 2009). However, the *in vitro* shoots of *T. peltatum* forming glandular leaves under P starvation, did not have any roots. Thus, the induction of carnivory is likely more related to the systemic response identified in *A. thaliana* as a second level, that is a systemic response to low internal Pi levels (Thibaud *et al.*, 2010). The Pi starvation response is characterised by changes in signal transduction, modifications of proteins and transcriptional reprogramming aiming to maintain Pi homeostasis with the Pi concentration and inositol phosphate being the important intracellular Pi sensing signals (Wang *et al.*, 2021). However, the exact mechanisms of signal perception and the coordination of the different transport systems on a cellular and subcellular level is not fully understood up to now (Wang *et al.*, 2021). The systemic Pi deficiency reaction also comprises effects on metal homeostasis (Thibaud *et al.*, 2010). Especially, iron was found to accumulate in *in vitro A. thaliana* seedlings raised under Pi deficiency (Hirsch *et al.*, 2006). Interestingly, also the Fe concentrations in *T. peltatum* shoots under P deficiency were significantly higher than in shoots from the control or from N and K deficiency (Table 5).

For *T. peltatum*, we observed the formation of carnivorous leaves in response to Pi starvation to start not synchronously and not in all shoots. This may be due to differences in P concentrations, fresh mass of the starting material and growth rates, but also in the P distribution within the shoots. Earlier formation of carnivorous leaves in some experiments was most likely due to lower P concentrations of the starting material: 6 months after the last subculture, the shoots were already depleted in P and contained 0.95 mg g^{-1} DM compared with the higher concentrations detected in shoots 2 months after subculturing (1.57 mg g^{-1} DM, Table 5). The gelling agent Gelrite provided 0.14 mM P in the solidified medium and was shown to vary considerably among batches. Liquid medium allowed to better define the P content of the medium and revealed that nutrient solutions with 0.2 mM P or lower resulted in the formation of glandular leaves. Future research should address the time course of Pi uptake and employ techniques to analyse P contents in the shoots with a spatial and chemical resolution (Wieczorek *et al.*, 2022).

Among carnivorous plants, *T. peltatum* has a unique strategy in mineral nutrient acquisition

Carnivorous plants are evolutionarily approved to cope with the nutrient limitation of their habitats (Adamec, 1997; Ellison, 2006). In general, outgrowth of traps on carnivorous plants is part of the developmental process, in other words it is hardwired. They represent modified leaves capable to capture and consume prey derived nutrients. In the Venus flytrap, which is equipped with active traps, mechanical and chemical senses report about the presence of animal food stocks. In turn, digestive enzymes are produced that decompose the prey and transporters

are activated to take up the nutrients released (Scherzer *et al.*, 2013, 2015; Paszota *et al.*, 2014; Bemm *et al.*, 2016; Böhm *et al.*, 2016). To take advantage from P in an animal meal, the expression and activity of organic phosphatases and Pi transporter is induced (Schulze *et al.*, 2012).

Triphyophyllum peltatum does not develop and energise cost-intensive traps under sufficient P supply. As a liana, it has to grow fast and climb up to the top of the rain forest for proper photosynthesis. In the facultatively carnivorous plant *T. peltatum*, P besides representing a nutrient, serves as a signal. Under episodes of soil P depletion when the intracellular Pi concentration drops below a critical threshold, *T. peltatum* invests in the formation of carnivorous leaves to be able to complement their P stocks from captured animals. After soil Pi content recovers to prestress levels, 'cheaper' photosynthetic leaves (Ellison, 2006) get produced.

Future directions

Having established the *in vitro* and *ex vitro* cultivation of *T. peltatum* and induction of carnivorous leaves, future genome sequencing and analysis of RNA profiles from P starvation-induced leaves together with the related pre- and poststimulation scenarios will provide important insight into the molecular mechanism of facultative carnivory in terms of the optimisation of fitness cost. Understanding the proximate and evolutionary basis of diet-induced plasticity is important because such plasticity might play an underappreciated role in the origins of diversity in botanical carnivory.

One of the unique properties of *T. peltatum* is the fact that among the numerous known carnivorous plants, it is the only taxon that produces naphthylisoquinoline (NIQ) alkaloids (i.e. the only polyketide-derived isoquinolines ever found in nature) – and, vice versa, among the NIQ-producing plants it is the only one to be carnivorous, thus occupying an extraordinary position at the intersection between carnivory and NIQ production. It will be of interest to explore a possible interdependence of these two phenomena from each other at the molecular level.

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Competing interests

None declared.

Author contributions

TW was responsible for the design of the research strategy, data collection and analysis, and wrote the manuscript with the help of RH, GB and AH. TW (*in vitro* rooting, *in vitro* induction of carnivory, *ex vitro* induction of carnivory), AH (nutrient analyses) and GB (provision of plant material) were involved in research experiments. TW and RH were involved in data interpretation. All authors read and approved the final manuscript.

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Data availability

All data are available at the Research Data Repository of Leibniz Universität Hannover under the following DOIs: 10.25835/1qnvahnm (Winkelmann & Herwig, 2023); 10.25835/t359h7o4 (Winkelmann, 2023a); 10.25835/9jnx1x1b (Winkelmann, 2023b).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Mean number of roots per rooted *in vitro* shoot of *Triphyophyllum peltatum* in eight rooting experiments.

Fig. S2 Length of the longest root of *Triphyophyllum peltatum in vitro* shoots in eight rooting experiments.

Fig. S3 Transitional leaves and reversion to normal leaves 4 wk after transferring the shoot P deficiency to control medium.

Fig. S4 Development of glandular leaves depending on the cultivation time on P deficiency medium.

Fig. S5 Percentage of shoots forming glandular leaves *in vitro* over time in liquid medium with different Pi concentrations.

Fig. S6 *Triphyophyllum peltatum* shoots after 21 wk of culture in liquid medium containing different Pi concentrations.

Fig. S7 Two 3-month-old *Triphyophyllum peltatum* plants fertilised with or without P.

Fig. S8 Development of glandular leaves on glasshouse-grown plants over time.

Notes S1 Analysis of phosphorus contents in different batches of Gelrite.

Table S1 Experimental treatments tested to induce glandular leaves *in vitro*.

Table S2 Composition of liquid media for induction of carnivorous leaves *in vitro*.

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