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Experimental field cultivation of *in vitro* propagated high-yield varieties of *Tropaeolum majus* L.

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Summary

About 10,000 mass propagated clonal progenies of the medicinal plant *Tropaeolum majus* L. had been cultivated in an experimental field trial to analyze the large scale cultivation of nasturtium-plants for pharmaceutical utilization. The glucotropaeolin contents of the eight *Tropaeolum*-clones, which had been established and propagated by *in vitro*-culture techniques, had been monitored and compared with unselected plants from commercial seed mixtures (*sm*-plants). Whereas the intra-clonal variation of the glucosinolate levels was significantly lower than the variability of the *sm*-plants, the glucotropaeolin content in the clonal progenies was markedly lower than in both, in the clonal mother plants as well as in the *sm*-plants. The proposed explanation for this phenomenon is based on the fact that the genetically identical cloned plants reveal only a very narrow phenotypical amplitude, which accordingly resulted in designated glucosinolate levels due to the certain environmental situations. However, under changing conditions, the corresponding glucotropaeolin content might be much lower. In contrast, the *sm*-plants reveal – due to the strong genetic heterogeneity – a much broader phenotypical amplitude of their physiological characteristics. Consequently, under changing growth conditions various individual plants may accumulate high amounts of glucotropaeolin. These coherences explain both, firstly, the finding that the clonal mother plants revealed very high glucotropaeolin levels under the certain – maybe spatial limited cultivation conditions – whereas their progenies accumulate far less glucosinolates; and secondly, that the average content in the *sm*-plants is higher than the mean content of the clonal progenies.

These data suggest that the much cheaper growing of nasturtium plants from seeds should be favoured over the more sophisticated *in vitro*-propagation techniques. Anyhow, for industrial farming there is one great advantage for the usage of *in vitro* generated *Tropaeolum* plants: the selected, *high glucosinolate*-nasturtium clones all reveal a compact growth with short tendrils. Therefore, the mechanical harvest of the corresponding clonal progenies, is quite unproblematic in comparison to the difficile harvest of *sm*-plants, most exhibiting tendrils of several meters.

Introduction

Nasturtium (*Tropaeolum majus* L.), a traditional medicinal plant containing high amounts of glucotropaeolin is used to treat infections of the urinary tract (Hoffmann-Bohm and Koch, 1994). In contrast to most other glucosinolate plants *T. majus* contains just one type of glucosinolate (Kjær et al., 1978), i.e. the benzylglucosinolate, also named glucotropaeolin. In the course of any decompartmentation, e.g. during herbivore feeding or pathogen attack, glucotropaeolin is hydrolyzed by myrosinases to yield the unstable thiohydroximate-O-sulphonates. Depending on the reaction conditions (i.e. pH, the presence of Fe^{2+}-ions, or the abundance of epithiospecifier proteins) these compounds react to a wide array of further products, the so-called mustard oils, comprising of isothiocyanates, nitriles or thiocyanates (for review Bones and Rossiter, 2006; Halkier and Gershenzon, 2006; Selmar, 2008), revealing important functions within the various ecological interactions, e.g. as protective agent (for review see Wittstock et al., 2003; Wheat et al., 2007). When *Tropaeolum* leaves are consumed, the mustard oils produced are absorbed in the intestine and finally excreted into the urine (Mennicke et al., 1988; Vermeulen et al., 2003). Due to the antimicrobial activity of the benzyl isothiocyanate, the growth of bacteria causing the inflammations is inhibited and the infection of the urinary tract (e.g. cystitis) is cured (Hoffmann-Bohm and Koch, 1994). Recently, various efforts had been made to use dried nasturtium leaves as modern phytopharmacon (Kleinwächter 2008; Kleinwächter et al., 2008). For this purpose, a sufficient high glucotropaeolin concentration in the dried drug is required, i.e. a very high glucosinolate concentration in the fresh leaves as well as a minimal loss whilst drying. A corresponding screening of several hundreds individual nasturtium plants resulted in the selection of eight promising clones from which *in vitro*-plants had been generated and micro-propagated (Kleinwächter et al., 2008). Corresponding progenies had been transferred into soil and were cultivated in a comprehensive field trial (Fig. 1). In this paper, the glucotropaeolin contents of these nasturtium plants are presented and compared with those of the mother plants in order to create a solid basis for the large scale cultivation of *Tropaeolum*-plants for pharmaceutical utilization.

Materials and methods

Plant material

From the plants selected by an extensive screening described by Kleinwächter et al. (2008) *in vitro*-plants plants have been generated and propagated on 0.8 % agar with MS-media (Murashige and Skoog, 1962), containing 0.1 ppm TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl-urea) and 0.5 ppm IAA (3-indole acetic acid); for details see Matallana et al. (2006). After mass propagation the *in vitro*-plants (Fig. 1a) had been transferred into soil. After eight weeks of acclimatization in a green house (Fig. 1b), over 10,000 progenies were planted on an experimental field with about 60 cm spacing (Fig. 1c). For fertilization, 100 kg N / ha (as calcium/ammonia nitrate) and 100 kg S / ha was applied as elemental sulphur (Kumulus, BASF). In total, an experimental area of nearly one half hectare was used for cultivation of *Tropaeolum* (Fig. 1d). Three months later leaves were harvested. The plants derived from the commercially available seed mixture (SM) were sowed by applying six seeds per hole about three weeks before the *in vitro*-plants were transferred in the field as described by Kleinwächter et al. (2008).

Sample preparation

Tropaeolum leaves were harvested while the plants were flowering. For quantification of glucosinolates in the fresh leaves, directly after

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detaching the leaves – still in the field – the plant material was shock frozen in liquid nitrogen. To exclude any intra-individual variations, in each case 15 leaves of each plant were pooled as one sample. The material was homogenized with mortar and pestle in liquid nitrogen, and subsequently freeze dried. 20 mg aliquots of the lyophilized materials were used for the glucotropaeolin analyses. Each quantification was performed as independent double estimation.

Quantification of glucotropaeolin

Quantitative analyses of glucotropaeolin were performed by HPLC: 160 µL arbutin (10 mM) were added as internal standard to each 20 mg-aliquot. The samples were extracted three times with 1 mL of MeOH (80 %, containing 8.5 mM ammonia acetate). Extraction was boosted using ultrasonification (10 min at 50°C). After centrifugation (15 min at 10.000 x g), the supernatants were pooled and concentrated by evaporation to final volumes of about 200 to 300 µL. After the estimation of the exact volumes, water was added to yield exactly 600 µL of aqueous samples. Then 1280 µL ammonia acetate (43.5 mM) and 120 µL MeOH were added to obtain a final volume of exactly 2 mL and to achieve the composition of the HPLC eluent A (see below).

HPLC analysis was performed using a RP 18 column (250 x 4 mm). Elution (1 mL / min) was achieved by applying a one step gradient 8 min eluent A followed by 3 min eluent B (A: 6 % MeOH, 40 mM ammonia acetate, B: 14 % MeOH, 40 mM ammonia acetate). For the detection of glucotropaeolin and arbutin absorbance was recorded at 230 nm. In order to eliminate all undesired substances from the column, after each chromatography, a rinse cycle with 80 % MeOH (10 min) and a re-equilibration step (25 min, eluent A) were introduced. Based on the peak areas of glucotropaeolin and the internal standard, the amounts of glucotropaeolin were calculated.

Results and Discussion

Variation of glucotropaeolin content of nasturtium plants

In order to screen for glucotropaeolin-rich *Tropaeolum*-varieties exploitable for pharmaceutical usage more than 200 individual plants had been analyzed by KLEINWÄCHTER et al. (2008). This screening

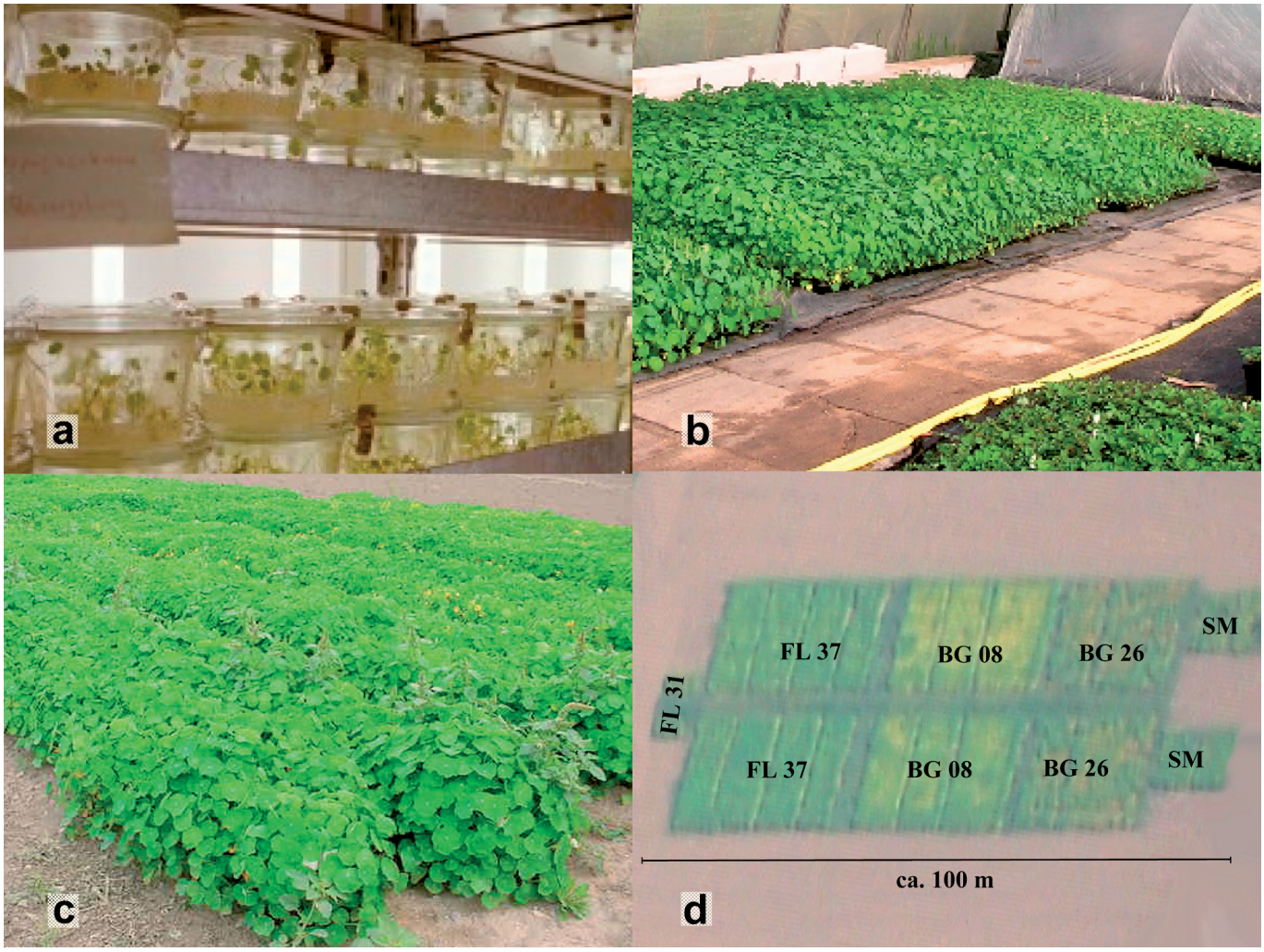

Fig. 1: Mass propagation of *Tropaeolum majus*.
a) large-scale *in vitro*-propagation of the eight selected varieties in the growth chamber.
b) acclimatization of the progenies in the green house.
c) field trial of FL 37: like the other clones, also this variety exhibits a compact growth with short tendrils.
d) aerial view of the entire field trial 2005.

revealed that the glucosinolate content in the leaves varied drastically from less than 40 µmol/g d.w. (~ 16 mg /g d.w.) to more than 130 µmol/g d.w. (~ 50 mg /g d.w.), corresponding to an average value of 60 µmol/g d.w. (Fig. 2). For the selection of suitable plants, special attention was paid to high glucotropaeolin content in the fresh leaves, to minimal losses of glucotropaeolin whilst drying and to compact growth characteristics (KLEINWÄCHTER et al., 2008). From the most promising plants eight defined clones had been established by *in vitro*-culture techniques. The question arose, if the glucosinolate levels of the genetically identical progenies of each clone would be similar to that of their mother plants, and if the variability of the glucotropaeolin contents might be less than that of the original, unselected nasturtium population. As expected, the intra-clonal variation was significantly lower than the variability of the unselected plants: in contrast to the high standard deviation of 32.4% (based on the average glucotropaeolin content for the original population of 60 µmol/g d.w., Fig. 2), it was markedly lower for the clonal progenies, being in the range from 8.8 to 15.2 % with an average value of only 12.0 % (Fig. 3). The same outcome is achieved when the highest divergences from the mean value are compared: this value is far over 110 % for the unselected mother plants and thereby nearly five times higher than for the clonal progenies.

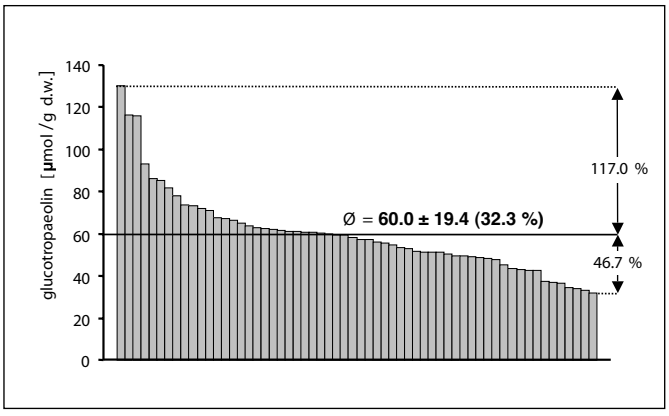

Fig. 2: Variation of glucotropaeolin contents of 60 individual nasturtium plants.
For the determination of the glucotropaeolin content, plants had been shock-frozen still in the field. Quantification was performed by HPLC. All values are based on at least two independent estimations, bearing maximal methodological variations of less than 1.5 %, average value was about 1 %. In order to illustrate the entire plasticity of the glucosinolate levels, the standard deviations as well as the maximal differences from the mean value (ø) are mentioned. 1 µmol glucotropaeolin per g dry weight (d.w.) corresponds to about 0,4 mg/g d.w.

When the mean values of the glucotropaeolin contents of the progenies are compared with those of the mother plants, it becomes obvious that the clones reveal quite lower glucosinolate levels than their genetically identical ancestors (Fig. 4). Yet, in this context it has to be considered that – due to the limited lifetime of the mother plants – the glucosinolate analyses pertain to plants which were grown in different, succeeding years. As the weather conditions had been quite different in the summers of the years 2004 and 2005, it can be assumed that either higher mean temperatures or less rainfall influenced markedly the growth as well as the glucosinolate content of the nasturtium plants; yet, these variables are not subject of this study and will be considered elsewhere. Similar effects were described by JENSEN et al. (1996), who found that the soil water potential influences the glucosinolate levels in rape seeds, and by ROSA (1997), who reported an corresponding impact of the temperature.

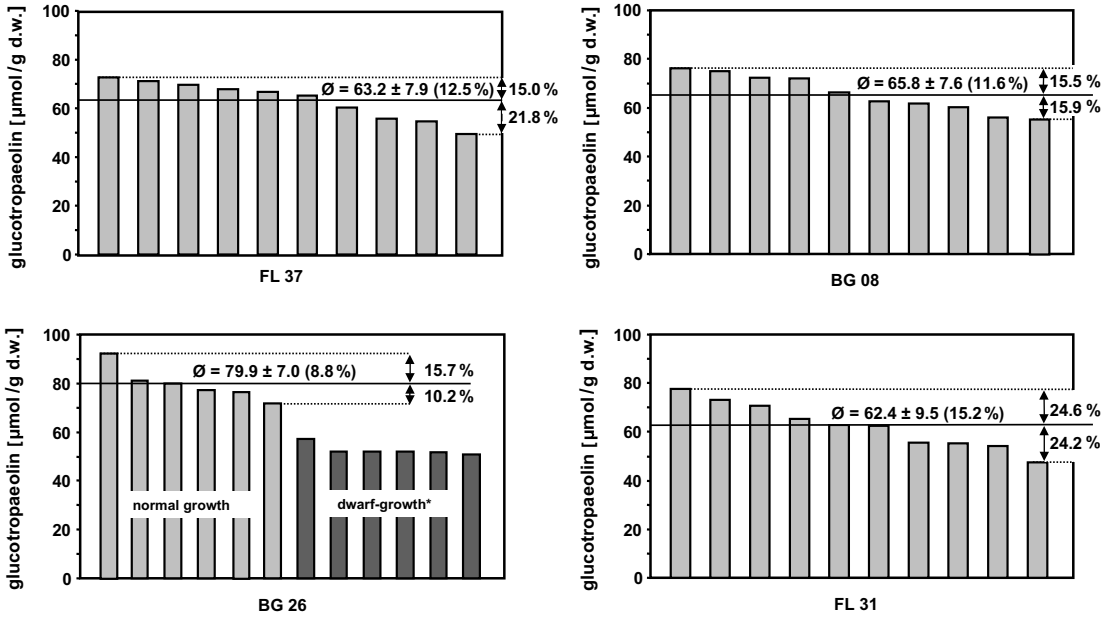

Fig. 3: Variation of glucotropaeolin contents of four nasturtium clones.
For the determination of the glucotropaeolin content, plants had been shock-frozen still in the field. Quantification was performed by HPLC. All values are based on at least two independent estimations, bearing maximal methodological variations of less than 1.5 %, average value was about 1 %. In order to illustrate the plasticity of the glucosinolate levels in each clone, the standard deviations as well as the maximal differences from the mean value (ø) are mentioned.
* The progenies of BG 26 revealed two quite different phenotypes: whereas ca. 55% of the BG 26-plants showed a normal growth, about 45% exhibited a reduced, dwarf-like phenotype.

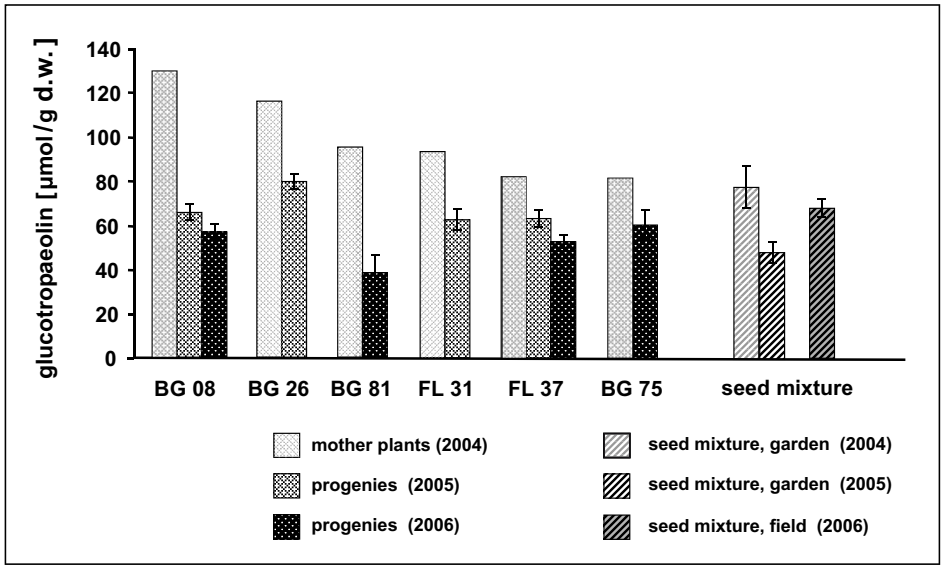

Fig. 4: The glucosinolate levels in mother plants, their clonal progenies and plants derived from seedlings.
For the determination of the glucotropaeolin content, plants had been shock-frozen still in the field. Quantification was performed by HPLC. In the case of the clonal progenies, each value corresponds to the mean value of 10 individual plants; each single determination is based on at least two independent estimations, bearing maximal methodical variations of less than 1.5%. Standard deviations are referred to the individual, clonal variability. In the case of the plants derived from the commercial seed mixture, 60 (2004), 50 (2005) and 15 individuals plants (2006), respectively, had been analyzed.

The assumption that the weather conditions indeed strongly influence the glucosinolate level also in *Tropaeolum* is underlined by the finding that 2004 also the glucotropaeolin content in the plants derived from the commercial seed mixture (*sm*-plants, grown in the botanical garden) is significantly lower than in the year 2005 (Fig. 4). Unfortunately, no corresponding data for the year 2006 are available. An analogous influence on the content of secondary plant products by various climatic factors had been reported frequently; in this context, water shortage and corresponding drought stress responses represent important parameters, known to modulate the content of glucosinolates (e.g. MAILER and CORNISH, 1987; JENSEN et al., 1996) and other natural products (for review see SELMAR, 2008).

Surprisingly, the mean glucotropaeolin-levels of the *sm*-plants (grown in the same experimental field as the clonal progenies) are markedly higher than that of the clonal progenies originated from the *high yield* mother plants (Fig. 4). These findings demonstrate that beside the weather conditions also other factors strongly effect the glucotropaeolin content. One possible explanation for this phenomenon is based on the fact that the genetically identical cloned plants reveal only a very narrow phenotypical amplitude, which accordingly – in a certain environmental situation – results in a designated high glucosinolate level. However, under other conditions, the corresponding glucotropaeolin content might be much lower. In contrast, the *sm*-plants reveal – due to the strong genetic heterogeneity – a much broader phenotypical amplitude and the physiological reactions might be very diverging. Hence it is very likely, that under different growth conditions, various individual plants respond with the accumulation of high amounts of glucotropaeolin. These coherences might explain both, firstly, the finding that the clonal mother plants revealed very high glucotropaeolin levels under the certain – maybe spatial limited cultivation conditions – whereas their progenies accumulate far less glucosinolates, and secondly, that the average content in the *sm*-plants is higher than that of the clonal progenies.

A further explanation for the observed differences in the glucosinolate levels of mother plants and their clonal progenies might be related to different basic growth characteristics: plants which had been developed from *in vitro*-plants reveal a different growth pattern than seedlings. Even after the required acclimatization of the propagated *in vitro*-plants for eight weeks in the greenhouse – compared to normal seedlings – the progenies showed an reduced growth for several weeks, when transferred into the field. In this context, the development of the root system could play an important role. A corresponding observation was made for the related *in vitro* plants: only under enhanced sulphur concentration in the medium, they reveal the same glucosinolate levels as the soil grown mother plants (MATALLANA et al., 2006), although the overall amount of sulphur available in the media is quite sufficient and in the same range as in the soil. From this it was deduced that the root system of *in vitro*-plants is not as effective as that of soil grown plants (MATALLANA et al., 2006). However, a corresponding initial difference in the root system of *in vitro*-plants and seedlings should be levelled during the course of the plant growth and development in the field.

Conclusion

The data presented in this paper demonstrate that the range of variation in glucosinolate content is far lower in the clonal progenies than in the plants originated from the standard seed mixture (*sm*-plants). However the mean glucosinolate contents are significantly lower than that of the *sm*-plants. Thus, the less cost-intensive growing of nasturtium plants from seeds should be favoured over the more sophisticated *in vitro*-propagation techniques. Anyhow, for industrial farming there might be one great advantage for the usage of *in vitro* generated *Tropaeolum*-plants. Beside the high glucosinolate levels, the nasturtium clones had also been selected for a compact growth, generating only very short tendrils. Therefore, the mechanical harvest of the corresponding clonal progenies, is quite unproblematic in comparison to the difficile harvest of plants exhibiting tendrils of several meters, which are frequently present in the sm-plants.

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