Elicitation, a New Window into Plant Chemodiversity and Phytochemical Drug Discovery

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Plant extracts collected from the wild are important sources for drug discovery. However, these extracts suffer from a lack of reproducible bioactivity and chemical composition caused by the highly inducible, variable, and transitory nature of plant secondary metabolism. Here, we demonstrate that exposing roots of hydroponically grown plants to chemical elicitors selectively and reproducibly induced the production of bioactive compounds, dramatically increased the hit rate, and more than doubled the number of plant species showing in vitro activity against bacteria, fungi, or cancer. Elicitation performed under controlled conditions dramatically improves reliability and efficiency of plant extracts in drug discovery while preserving wild species and their habitats.

Introduction

Plant natural products have been components of phytomedicines throughout human history. In the past hundred years, plants have become an important source for the discovery of novel pharmaceuticals, with many blockbuster drugs being directly or indirectly derived from plants.[1−3] However, the enthusiasm for using plant extracts for the discovery of novel pharmaceutical leads has been declining recently. Bioprospecting for plants and other organisms is losing out to high-throughput drug discovery, which relies more on combinatorial chemistry[4,5] and computational drug design.[6] Yet, it is believed that the majority of plant-derived natural products possibly valued at billions of dollars remain undiscovered or unexplored for their pharmacological activity.[7,8]

The lack of reproducibility of activity for more than 40% of plant extracts[2] is one of the major obstacles in using plants in pharmaceutical discovery, despite the great diversity of compounds they synthesize. The activities detected in screens often do not repeat when plants are resampled and reextracted. Moreover, the biochemical profiles of plants harvested at different times and locations vary greatly. This, in turn, creates a major difficulty for the prioritization, characterization, and isolation of active compounds. Also, complex plant extracts obtained from the above-ground parts collected from the wild complicate the determination of potency and novelty of the active ingredient, which is often present in trace amounts and obscured by pigments and polyphenols that interfere with many screens.

It is well-known that different stresses, locations, climates, microenvironments, and physical and chemical stimuli (often called elicitors) qualitatively and quantitatively alter the content of bioactive secondary metabolites. Enzymatic pathways leading to the synthesis of these phytochemicals are highly inducible.[9] This is particularly true for phytochemicals that are well documented for their pharmacological activity, such as alkaloids,[10] phenylpropanoids,[11] and terpenoids.[12,13] For example, the levels of phytoalexins, a large and structurally diverse group of antimicrobial plant defense compounds, often increase by 2−3 orders of magnitude following pathogen inoculation or elicitation.[14,15] In many cases, these compounds are nondetectable in the nonelicited plant tissues. Tissue culture and whole plant elicitation also increases the amounts of natural products widely used as pharmaceuticals, such as taxol,[16,17] tropane alkaloids,[18] indole alkaloids of *Catharanthus roseus*,[19,20] and salicylates.[21]

Massive qualitative and quantitative variations in the content of bioactive natural products were considered a detriment rather than an asset of phytochemical drug discovery and therefore never fully exploited in pharmaceutical bioprospecting. Applying elicitors onto the soil-grown plant has serious limitations associated with poor uptake of the chemical elicitors by the relatively impermeable, hydrophobic surfaces of plant shoots. Therefore, phytochemical elicitation as a discovery tool was only attempted in costly, inefficient, and difficult to maintain cell culture systems.[22]

Delicate and physically unprotected plant roots survive in a hostile soil environment that is teaming with bacteria, fungi, nematodes, and other herbivores. The largely unexplored chemodiversity of compounds from plant roots may harbor novel antimicrobial and anticancer compounds used by plants to destroy pathogenic microorganisms or selectively kill herbivores. While the biochemically active fibrous roots are difficult to harvest from the soil, roots can be easily grown in hydroponic systems under strictly controlled environmental conditions. Elicitors can be added to the medium and taken

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Table 1. Effect of Elicitation on the Anticancer Activity of Plant Root Extracts[a]

cancer type	no. of species tested	species with activity		species with activity in elicited samples ONLY		species with activity in nonelicited samples		species with activity in nonelicited samples ONLY	
		no.	%[b]	no.	%[b]	no.	%[b]	no.	%[b]
breast	588	80	14	63	11	17	3	7	1.2
melanoma	193	36	19	26	14	10	5	0	0.0
renal	193	26	13	16	8	10	5	1	0.2
CNS	335	49	15	27	8	22	6	6	1.0
lung	335	62	19	48	14	14	4	7	1.2
total[c]		119		76		43		11	

[a] All anticancer assays were based on growth inhibition of MCF, 7 (breast cancer); SF, 268 (CNS cancer); H, 460 (nonsmall cell lung cancer); UACC, 62 (melanoma); and TK, 10 (renal cancer). Plants were grown hydroponically under controlled conditions and elicited for 24 h by adding elicitors to the medium. [b] Percent of the total number of species (first data column) tested against the particular cancer cell line. [c] Total number of species found active against at least one cancer cell line. Numbers in this row are lower than the sum of the numbers in the corresponding columns, since these numbers count the same species several times if it is active against more than one cancer cell line.

up by the roots, which can be subsequently harvested and screened for bioactivity. Fortuitously, roots, in contrast to shoots, contain lower levels of pigments and other compounds that may interfere with screens and are much easier to grind and extract.

Our goal was to demonstrate that the 24-h-long elicitation of roots of hydroponically grown plants dramatically increases the frequency and reproducibility of early leads, with each elicitor triggering unique and specific antimicrobial or anticancer activities in plant roots. In an attempt to capture the greatest amount of biodiversity, 989 species belonging to 155 families were hydroponically grown, elicited, and assayed. Generally, data for fewer species are reported, since not every species was subjected to all treatments or screened against every target. To select the most potent and diverse elicitors, we screened 25 known bioactive compounds for their ability to elicit quantitative and qualitative changes in the biochemical composition of roots of three hydroponically grown plant species (see Supporting Information, Table 1). Acetate (0.1%), methyl jasmonate (0.1 mM), methyl salicylate (0.8 mM), and chitosan (0.1%) were found to be the most effective. The list of other tested elicitors can be found in the Supporting Information (Table 1). Some of these compounds were previously reported to have elicitor function in roots[23,24] or cell cultures.[25]

Results and Discussion

Effect of Elicitation on the Anticancer Activity of Plant Extracts. Of the 588 plant species chosen from a broad taxonomical background, 119 species had at least one anticancer activity in either elicited root extracts, nonelicited extracts, or both, producing an overall 20% hit rate (Table 1). Out of 119 active species, 39 were active against one cancer cell line, 25 against two, and 55 against three. While all species were tested against breast cancer cell line, fewer species were tested against cell lines representing the other four forms of cancer. Seventy-six elicited species had unique activity against at least one cancer cell line without detectible activity in the corresponding nonelicited samples. Samples from an additional 17 species were active against one cancer cell line only after elicitation, whereas nonelicited samples from the same species were inactive against this cell line. Data indicate that 64% of plant species (76 out of 119) would have been missed during the more conventional bioprospecting activity, leaving only 43 species active in the nonelicited state as potential sources of anticancer leads. The percentage of missed leads could be even greater if only one cancer cell line was used for screening (79% for breast cancer, 72% for melanoma, and 77% for lung cancer). Only 11 nonelicited species had activity against at least one cancer cell line, while none of the elicited samples from the same species were active. Nonelicited samples of two additional species were active against a particular cancer cell line, while the elicitation of the same species produced activities against different cell lines.

Effect of Elicitation on the Antimicrobial Activity of Plant Extracts. Elicitation also had a powerful effect on increasing the antibacterial and antifungal activity of root extracts. Of the 966 species tested, 49% (or 468) produced antimicrobial compounds in their roots (Table 2): 210 species were active against 1 microorganism, 133 were active against 2, 61 were active against 3, 46 were active against 4, and 18 were active against all 5. Elicitation doubled the number of species that showed general antimicrobial activity, effectively adding an additional 234 species to the list of antimicrobial plants. An additional 107 species were active against one particular microorganism only after elicitation, whereas the nonelicited root extracts from the same species were not active. Elicitation effects were even more dramatic for activity against a particular organism. Fifty-eight percent of the species active against *S. aureus* were active only after elicitation, 71% were active for *E. coli*, 71% were active for *P. aeruginosa*, 58% were active for *S. cerevisiae*, and 68% were active for *A. niger*. The largest percentage of plant species, 37%, were active against *S. aureus* (Gram-positive bacteria), while the smallest percentage, 12%, were active against *A. niger* (filamentous fungus). Extracts from 234 nonelicited species showed antimicrobial activity against at least one of the five tested microorganisms. Of these, the corresponding elicited extracts from 31 species were not active against any microorganism and 50 were active against a different microorganism.

Specificity of the observed biological activity was analyzed with 247 species treated with all four elicitors and assayed for both anticancer and antimicrobial activity. Out of these, 55 species had anticancer activity against at least one cell line (22% hit rate). Twenty-three species that were active against cancer cells did not have any antimicrobial activity, 11 had activity against *S. aureus*, 2 had activity against *E. coli*, 2 had activity against *P. aeruginosa*, 5 had activity against

Table 2. Effect of Elicitation on the Antimicrobial Activity of Plant Root Extracts[a]

microorganism	no. of species tested	species with activity		species with activity in elicited samples ONLY		species with activity in nonelicited samples		species with activity in nonelicited samples ONLY	
		no.	%[b]	no.	%[b]	no.	%[b]	no.	%[b]
S. a.	966	353	37	203	21	150	16	21	2
E. c.	966	153	16	108	11	45	5	16	2
P. a.	536	101	19	72	13	29	5	9	2
S. c.	966	202	21	117	12	85	9	23	2
A. n.	966	122	12	83	9	39	4	9	1
total[c]		468		234		234		31	

[a] Modified growth inhibition assays on solid agar medium inside 24-well culture plates were used to measure antibacterial and antifungal activity of plant root extracts. Microorganisms, three bacterial and two fungi, used for the bioassay were acquired from the ATCC: *S. aureus* subsp. *aureus* 6538 (*S. a.*), *E. coli* K-12 (*E. c.*), *P. aeruginosa* P-6 (*P. a.*), *S. cerevisiae* 99R (*S. c.*), and *A. niger* (ATCC 1015) (*A. n.*). Plants were grown hydroponically under controlled conditions and elicited for 24 h by adding elicitors to the medium. [b] Percent of the total number of species (first data column) tested against the particular microorganism. [c] Total number of species found active against at least one microorganism. Numbers in this row are lower than the sum of the numbers in the corresponding columns, since these numbers count the same species several times if it is active against more than one microorganism.

Table 3. Effect of Elicitors on Anticancer and Antimicrobial Activity of Plant Root Extracts[a]

	nonelicited	acetate	MeSA[b]	chitosan	MeJA[c]
anticancer breast, CNS, lung					
nonelicited	0, 1, 0				
acetate	0, 0, 0	8, 6, 8			
MeSA	0, 0, 0	1, 1, 1	1, 1, 2		
chitosan	0, 0, 0	0, 0, 0	0, 0, 0	1, 0, 0	
MeJA	0, 0, 0	0, 0, 0	0, 0, 0	1, 0, 1	0, 0, 1
antibacterial *S. a.*, *E. c.*, *P. a.*					
nonelicited	25, 17, 9				
acetate	23, 3, 4	76, 29, 13			
MeSA	2, 3, 1	11, 5, 3	14, 13, 22		
chitosan	3, 2, 0	14, 1, 2	4, 4, 1	14, 11, 9	
MeJA	3, 1, 1	7, 2, 1	3, 4, 2	7, 7, 4	14, 12, 13
antifungal *S. c.*, *A. n.*					
nonelicited	24, 9				
acetate	8, 7	35, 27			
MeSA	3, 3	9, 1	16, 11		
chitosan	2, 0	1, 1	3, 3	12, 13	
MeJA	2, 0	1, 2	2, 1	8, 4	13, 10

[a] Table entries refer to the number of plant species showing activity only after treatment with a particular elicitor or only after treatment with two different elicitors. Numbers are separated by commas, referring to activity against a specific cancer or microbial target shown in the order they are listed in the headingsSpecies: *S. aureus* subsp. *aureus* 6538 (*S. a.*); *E. coli* K-12 (*E. c.*); *P. aeruginosa* P-6 (*P. a.*); *S. cerevisiae* 99R (*S. c.*); *A. niger* (ATCC 1015) (*A. n.*). [b] MeSA: methyl salicylate. [c] MeJA: methyl jasmonate.

S. cerevisiae, and 2 had activity against *A. niger*. These data suggest that the observed activities were due to diverse bioactive compounds produced by different plants. Chromatographic data and activity-guided fractionation of active samples confirmed that the great majority of the observed activities are due to the plant-specific natural products produced in the roots of different plant species.[30] However, it is still possible that some plants metabolized the elicitors into biologically active compounds responsible for some anticancer or antimicrobial effects.

Relative Effectiveness of Elicitors. The analysis of the effects of different elicitors on plant species from Tables 1 and 2 demonstrates that for most targets acetate was more effective than other elicitors (Table 3). The table denotes plant species that produced root extracts active only after treatment with one or two elicitors in independent treatments or nonelicited control. Each elicitor was supplied separately to an independent set of plants. For example, the entry 0, 0, 0 in the second data row of the table means that no species showed activity in both acetate (row) and nonelicited treatments (column) against breast, CNS, or lung cancer lines. The 8, 6, 8 entry in the next column indicates that acetate made extracts from eight species active against breast cancer lines, six species active against CNS, and eight species active against lung, while no other elicitors were effective with these species. To save space, the activity relation between three or more treatments is not shown. The effects of acetate and other elicitors were not simply a result of changes in the solution pH.[30] More plant species showed activities exclusively after acetate treatment than after any other treatment, including nonelicited controls. However, methyl salicylate was more effective than acetate, and methyl jasmonate was equal to acetate in inducing activity against *P. aeruginosa*. We did not observe any two of the five treatments forming activity grouping whereby the activity of one elicitor will positively or negatively correlate with the likelihood of the other being active against a particular target.

Taxonomic Distribution of the Elicitation Effect. The representative plant families demonstrating the general trends in the selectivity and effectiveness of elicitation are shown in Table 4. For example, following various treatments, some species from *Anacardiaceae* and *Apiaceae* were able to produce antimicrobial compounds but not anticancer compounds. Conversely, a large proportion of active species from *Asteraceae, Caryophyllaceae, Cucurbitaceae,* and *Polemoniaceae* produced anticancer compounds. While in most of the families acetate was the most effective elicitor of bioactivity, it was not the case for the *Polemoniaceae*

Table 4. Effect of Elicitation on Bioactivity of Plants Belonging to Different Families (Also See Table 2 in Supporting Information)[a]

family (tested species/active species)	activity	elicitors					
		acetate	chitosan	MeJA	MeSA	ne	total activities
Anacardiaceae (3/3)	AB	2	3	3	3	2	13
	AF						
	AC	3	1	1			5
Apiaceae (6/5)	AB	2	1	2	1	2	8
	AF	1		1		1	3
	AC						
Asteraceae (20/14)	AB	9	1	2		2	14
	AF	5	1	1	1	1	9
	AC	8	1		1	2	12
Brassicaceae (12/7)	AB	3		1	3	4	11
	AF					1	1
	AC	2	1	1	1	1	6
Caryophyllaceae (4/4)	AB	1			1	1	3
	AF	1		1	2	1	5
	AC	2	1		2	1	6
Cucurbitaceae (4/4)	AB	1			1		2
	AF						
	AC	4	3	3	3	3	16
Fabaceaey (22/13)	AB	8	6	3	3	2	22
	AF	1	3	3	2	1	10
	AC	4	2	1	1	1	9
Lamiaceae (31/10)	AB	4	3	1	1	1	10
	AF		1				1
	AC	3	1		2		6
Polemoniaceae (5/3)	AB		1	1			2
	AF	1	1	1	1	1	5
	AC	1	1	1	1	1	5

[a] Roots were exposed to five treatments, four elicitors, and one nonelicited control (ne). Numbers in parentheses denote total number of species in each family subjected to all five treatments over the number of species in that family showing at least one activity in antibacterial (AB), antifungal (AF), or anticancer (AC) screens. Numbers in the table refer to number of species having a particular activity following treatment with a particular elicitor.

species. The complete list of the species summarized in Table 4 can be found in the Supporting Information (Table 2).

Conclusions

The results demonstrate a major stimulatory effect of elicitation on the production of bioactive compounds in hydroponically grown plant roots and the impact this technology may have on the early lead detection process. Drug discovery from plants customarily operated in a two-dimensional space whereby different plant species were assumed to contain different bioactive compounds, thus, ignoring the fact that biosynthesis of many bioactive secondary metabolites is highly inducible. The goal of bioprospecting has always been to screen as many species as possible in order to generate a maximum number of leads. Our results suggest that specific treatments compel each plant to generate a much greater number of leads in a more reliable and reproducible fashion. Thus, the controlled elicitation of roots of hydroponically grown plants adds an essential third dimension that affixes the chemodiversity of a particular plant species at a defined point. This in turn allows a much more efficient characterization and exploitation of the biochemical space encoded by plant genomes. Extensive LC−MS analysis of root extracts confirmed that elicitation induced major qualitative and quantitative biochemical changes in the chemical composition of roots. These changes are specific for each elicitor and species.[30] Chromatographic fingerprints induced by different elicitors in a single species were often as different as fingerprints of unrelated species. Cell cultures of plants also respond to elicitors. However, hydroponic cultivation is fast, simple, and applicable to a great majority of plant species, whereas cell cultures of most species are more difficult to produce and maintain using available technologies.

Most notably, the effects of elicitation on the chemical composition and bioactivity could be readily reproduced if the plants were regrown and reelicited under the standard greenhouse conditions used in this study. We were able to repeat 85% of antimicrobial activities initially observed in the screens. Lack of reproducibility for the remaining 15% may be attributed to the degradation of some active compounds during prescreening storage. Elicitation combined with controlled cultivation overcomes the major limitations of plant samples collected from the wild. It increases the bioactivity and reproducibility of plant extracts and allows a more efficient search for novel pharmacologically active plant natural products. Clearly, genotypic variations observed in different plant populations imposed both substantial variation and a genetic limit on the production of bioactive compounds. However, elicitation may be able to increase the production of some bioactive compounds up to the genetic limit. Laboratory- and greenhouse-based elicitation technology also preserves wild habitats and endangered species from being depleted by unscrupulous collectors.

Experimental Section

Plant Cultivation. The seeds obtained from the commercial seed companies or botanical gardens were germinated in a greenhouse inside a 0.9 cm in diameter, 0.9 cm deep well cut into rockwool cubes (3.4 cm width × 3.4 cm length × 3.7 cm height; Grodan, Hedehusene, Denmark). These cubes were placed inside standard greenhouse plastic trays (52 cm length × 25 cm width × 7 cm height) and watered with an overhead misting system. Seeds were allowed to germinate for 10−15

days until the roots started to emerge from the bottom of the rockwool cube. Plants were kept in the environmentally controlled greenhouse under a 16 h photoperiod maintained with supplementary lighting and at a temperature of 21 ± 2 °C at night and 26 ± 2 °C during the day. The rockwool cubes with seedlings were inserted into 12 precut circular holes (3.8 cm diameter) each in polystyrene foam rafts (25 cm length × 20 cm width × 3.8 cm height), which were floated on 7 L of hydroponic nutrient solution [2 g/L Hydro-Sol (Scotts-Sierra Horticultural Products Co.) supplemented with 1.5 g/L $Ca(NO_3)_2$ and 0.083 g/L NH_4NO_3] contained inside 10.8 L polyethylene pans (34 cm length × 29 cm width × 13 cm height). Hydroponic solutions were aerated by sparging 100 mL/min compressed air through plastic tubing ending with several nozzles.

Elicitation. Seedlings were cultivated hydroponically 4−6 weeks with roots growing in the aerated nutrient solution. Thereafter, plant roots rinsed with tap water and the polystyrene foam rafts were placed into 3 L aluminum trays (32 cm length × 26 cm width × 6 cm height) containing 400 mL of distilled water only or with an elicitor. To avoid excessive pressure on the roots, metal brackets were used to support the polystyrene raft above the solution. To prevent water loss from the leaves and drying of the solution, plant shoots were covered with transparent plastic bags. During elicitation, containers with plants were aerated by shaking at 40−45 rpm on a gyratory platform shaker (3590 Lab-line Barnstead International). Elicitors were used at the final concentrations of 0.1% acetic acid, 0.8 mM methyl salicylate, 0.1% soluble chitosan, and 0.1 mM methyl jasmonate.

Extraction and Sample Preparation. After 24 h of exposure to each elicitor, the roots were excised, rinsed with distilled water, blotted with filter paper, weighed, frozen at −80 °C, and freeze-dried. Freeze-dried roots were ground with a glass rod and extracted with 80% methanol (20 mL per gram of roots) at room temperature for 48 h on a gyratory shaker adjusted to 80 rpm. The insoluble materials were separated by centrifugation at 500*g* for 30 min. The extracts were decanted and vacuum-dried in a Savant AES 2010 vacuum centrifuge. The dry extracts were kept at −20 °C for bioassay and analytical studies. For antimicrobial screening, 15 mg/mL of dry plant root extract in DMSO was prepared.

Preparation of the Microorganisms. Bacterial cultures were grown on solid agar media (LB Agar, Miller, Fisher Scientific). Before screening, bacteria were transferred into liquid media in 125 mL flasks and cultivated overnight at +37 °C on a gyratory shaker (model G10, New Brunswick Scientific Co.) at 120 rpm. *Saccharomyces cerevisiae* was treated the same way as bacteria except that initial cultures were cultivated on potato dextrose agar media (Difco Laboratories) and transferred to potato dextrose liquid media 1 day before screening. The optical densities of all microorganism suspensions were measured on a Beckman DU 640 spectrophotometer at 560 nm. Suspensions of microorganisms were used in the screens after they reached the following optical densities: *Escherichia coli*, 0.015−0.030; *Staphylococcus aureus*, 0.01−0.02; *Pseudomonas aeruginosa*, 0.01−0.02; *S. cerevisiae*, 0.5−0.7. Spores of *Aspergillus niger* plated on potato dextrose solid agar media were collected and immediately replated for screening.

Antimicrobial Screens. Modified growth inhibition assay on solid medium was used to determine antibacterial and antifungal activity of plant root extracts. Sterile 24-well culture plates (Greiner Labortechnik) were filled with 1 mL/well of nutrient LB agar media, which was used for both bacterial and fungal bioassays. An amount of 10 μL of DMSO-dissolved extract was added to the surface of the agar media. After drying for 5−10 min, 30 μL of microorganism suspension was uniformly plated into each well. Spores of *A. niger* were applied on the surface of the media using a sterile applicator.

All samples were plated in triplicate plus the control. After 24 h (48 h for *A. niger*) of incubation at +30 °C in an incubator (Isotemp, Fisher Scientific), plates were examined and the antimicrobial activity was visually scored. Activity was rated from 1 to 5, 1 being the lowest and 5 being the highest growth inhibition.

Anticancer Screens. All anticancer assays were performed by the NCI, Division of Cancer Treatment and Diagnosis, Developmental Therapeutics Program, as described earlier[26−29] and as available on http://dtp.nci.nih.gov/branches/btb/ivclsp.html.

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Supporting Information Available: Complete list of all tested elicitors (Table 1) and all plant species summarized in Table 4 (Table 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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