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REVIEW

Forever young: stem cell and plant regeneration one century after Haberlandt 1921

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Abstract

Plants are characterized by a post-embryonic mode of organ development, which results in a need for these photoautotrophic organisms to regenerate lost parts in the course of their life cycle. This capacity depends on the presence of "pluripotent stem cells," which are part of the meristems within the plant body. One hundred years ago, the botanist Gottlieb Haberlandt (1854–1945) published experiments showing wounding-induced callus formation, which led ultimately to plant regeneration in tissue culture and thence to the techniques of "plant biotechnology," with practical applications for mankind. Here, we recount Haberlandt's discovery within the context of his long research life and his most influential book *Physiologische Pflanzenanatomie*. In the second part, we describe and analyze a plant tissue-culture regeneration system using sterile, dark-grown sunflower (*Helianthus annuus*) seedlings as experimental material. We document that excised hook segments, which contain a "stem cell niche," can regenerate entire miniature *H. annuus*–plantlets that, raised in a light/dark regime, develop flowers. Finally, we discuss molecular data relevant to plant regeneration with reference to phytohormones and conclude that, one century after Haberlandt, 1921, the exact biochemical/genetic mechanisms responsible for the capability of stem cells to remain "forever young" are, although already complex, really just beginning to become known.

Keywords Haberlandt · Stem cells · Plant regeneration · Auxin · Phytohormones

Introduction

Twelve years ago, Vasil (2008) summarized the history of plant biotechnology, an agenda of applied research that "came of age" about 25 years ago with "the planting of nearly five million acres of biotech crops, mostly in the United States." Maize (*Zea mays*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), and canola (*Brassica napus*, etc.) are the most important biotechnologically improved plant species grown with large commercial success. The cultivation of these (and other) biotech crops led to a reduction in the application of agrochemicals, contributed to an enhancement in productivity and economic development, and may even have improved human health. Under the headline "The Cell Theory," Vasil (2008) pointed out that the experimental foundation of modern "green biotech" rests on two key discoveries: Cellular *totipotency*, and the genetic transformation of crop plants. Whereas the methods of genetic modification by the uptake, incorporation, and expression of foreign genes (for instance, via the *Agrobacterium* technique) have been described in many books and articles, the "first principle" of this system of methods for improving plant productivity has not yet been analyzed in such detail (Vasil 2008). Totipotency is a property of plant stem cells, which can be defined (for embryophytes) as "active groups of pluripotent cells embedded in specialized tissues called meristems" (Greb and Lohmann 2016). These authors have pointed out that, in long-lived woody plants, plant stem cells can remain active for thousands of years, so

Dedicated to the memory of Winslow R. Briggs (1928–2019).

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it is appropriate to use the term "forever young" to characterize this property.

It is well known that Gottlieb Haberlandt (1854–1945) (Fig. 1) was the first to conceive of, and to suggest possible ways to investigate, what later became called totipotency of plant cells: the ability of a non-reproductive cell to produce, by repeated divisions, a whole plant (Höxtermann 1997). In the present contribution, which commemorates the 100th anniversary of one of Haberlandt's key discoveries pertinent to stem cells/plant regeneration, i.e., wound stress–activated cell division, we recount the life and achievements of this outstanding biologist. Since, in the year 1921, Haberlandt began to prepare the 6th and final version of his most important book *Physiologische Pflanzenanatomie* (Physiological Plant Anatomy), which edition was published in 1924, we also discuss the significance of this seminal monograph. In this context, we document that, in etiolated sunflower (*Helianthus annuus*) seedlings, the hypocotyl hook represents an as yet unidentified "stem cell-niche" and describe a system for plant regeneration of this important crop species.

From plant anatomy to cell cultures

The distinguished late nineteenth- or early twentieth-century botanist Gottlieb Haberlandt is probably best remembered today for the dramatic advances in plant-biological thinking that he achieved in his textbook *Physiologische Pflanzenanatomie* (Physiological Plant Anatomy). In this monograph, Haberlandt (1884; 6. ed. 1924) deduced functional roles for many of the anatomical structures, which improvements in the compound microscope during the nineteenth century had enabled plant anatomists to recognize and name. However, during his research career, one of his sustained efforts was to try to grow plant cells in isolated culture. His aim was to demonstrate (*inter alia*) if any of them are what we now call "totipotent," that is, capable of giving rise to cells, tissues, or structures having any of a species' differentiated cellular characteristics other than its own, or indeed develop into a complete plant among whose cells all these characteristics would occur. As a relatively young professor at the university in the Austrian alpine city of Graz, he first broached this forethoughtful concept in a 1902 paper (Haberlandt 1902a). After years of work which did not quite succeed in achieving true plant cell cultures, but led to important new concepts, such as that of plant hormones controlling cell division (Höxtermann 1997), he presented the evidence for this in several papers just one century ago (Haberlandt 1921a, b) and in the following few years (Haberlandt 1922, 1925). With all the experimental attention that has been given to plant tissue and cell cultures and their totipotency in the subsequent 100 years (Vasil 2008), it seemed appropriate, in a paper like this one, to remember Haberlandt's foresight about it.

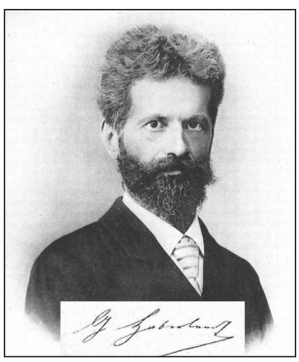

Fig. 1 Portrait of the botanist Gottlieb Haberlandt (1854–1945). His signature was added to the photograph (from Guttenberg 1955)

Physiological function of cells and tissues

Although he ultimately received what was then doubtless the most prestigious German professorship of botany, that in Berlin, Gottlieb Haberlandt was actually born (1854) far away in the town of Ungarisch-Altenburg (now known as Mosonmagyaróvár) near Vienna, but in the Hungarian part of the then-existing Austro-Hungarian empire (Noé 1934). His father taught natural sciences at the local agricultural school, whence Gottlieb already at a young age had acquired some exposure to biology. He early showed a talent for drawing and painting, which lasted actively throughout his life both recreationally (landscapes, portraits) and professionally (innumerable drawings of the cellular details of plant-anatomical structures as seen with the microscope, many published in his scientific books and articles). Examples of Haberlandt's scientific art capabilities are given in Figs. 2, 3, 4, and 5, where we show a series of his drawings.

At age 19 (1873), Haberlandt entered nearby Vienna University, where his interest was drawn to botany by Julius Wiesner's (1838–1916) lectures on plant anatomy and physiology, and by the friendly interest that this professor took in him. He started into plant-anatomical research forthwith, publishing his first botanical paper, in the *Österreichische Botanische Zeitung* (Austrian Botanical Journal) the very next year (1874), a study of the cellulose in cork, the material in which biological cells had first been seen and named (by Robert Hooke in 1665).

Vienna University awarded Haberlandt a PhD in 1876, for a thesis that investigated the wintertime coloring pigments of evergreen leaves. He then went to Tübingen University in southern Germany for a postdoc period with Professor Simon Schwendener (1829–1919), one of the outstanding German-speaking botanists of his day (he was actually Swiss). While there, Haberlandt became acquainted with Charlotte Haecker (1858–1911), to whom he became engaged and, as we shall see, later married.

Inspired by Schwendener's then-recent book *Das mechanische Prinzip im anatomischen Bau der Monocotylen*

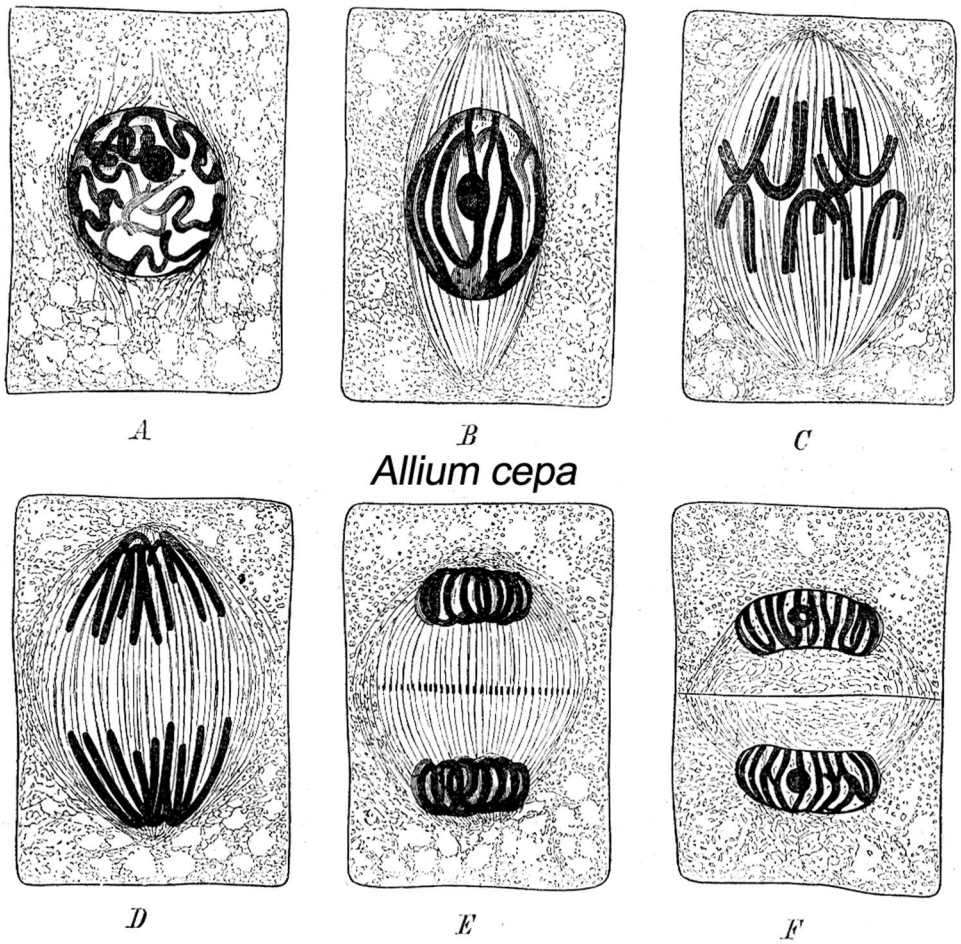

Fig. 2 Mitotic nuclear division (**A**–**E**) and subsequent cell division (**F**) in apical meristem of an onion root. This tissue represents a stem cell niche that continuously produces new daughter cells (from Haberlandt 1924)

(Mechanical Principles in the Anatomical Structure of Monocotyledons) (Schwendener 1874) and this professor's long-continued interest in finding out how plant-anatomical structures satisfy plants' needs for mechanical support with a minimum of cost to the organism, Haberlandt conceived the idea of making a systematic study of the physiological functions of plant-anatomical structures, and in Tübingen he undertook its first part, "mechanical tissue systems." He turned next to the chlorophyll-bearing photosynthetic systems and pursued this and subsequent tissue systems while becoming (in 1879) a lecturer in botany back at the University of Vienna, and then (1880) in the university in the Austrian alpine town of Graz. There he worked up through the ranks to become full Professor of Botany in 1888. In that capacity, he was finally (in 1899) able to solve the prolonged shortage and cramped character of botanical research facilities at Graz by getting built an attractive new Botanical Institute building which, as a professor, he would be able to direct.

The year after arriving in Graz (1881), Haberlandt married Charlotte Haecker. They had almost 30 happy years there together, and 5 children, in the beautiful town and alpine surroundings of Graz. She sadly died early in 1911, shortly before they were to have moved away to Berlin (see below).

At the time (1878) that Haberlandt left Tübingen, Prof. Schwendener also left, having been offered the professorship of botany in the University of Berlin. There he established a well-equipped research laboratory which soon had very capable graduate students tackling plant-anatomical problems with physiological or biophysical overtones, like those that Haberlandt had embarked on investigating systematically in his physiological plant-anatomical survey project mentioned above. He thus apparently began to feel pressure to publish a preliminary version of this project even though he had thus far looked relatively cursorily into many aspects of plant structure that he intended to cover eventually in greater detail. In 1884, he thus came out with the book *Physiologische Pflanzenanatomie im Grundriss dargestellt* (Physiological Plant Anatomy, Presented in Outline). This became the first edition of his long-enduring masterpiece (Haberlandt 1884) that went through no less than 6 successive editions of enlargement and revision, those beyond the first one lacking the "in outline" subtitle, and the 3rd one (1904) receiving a complete translation into English (in 1914).

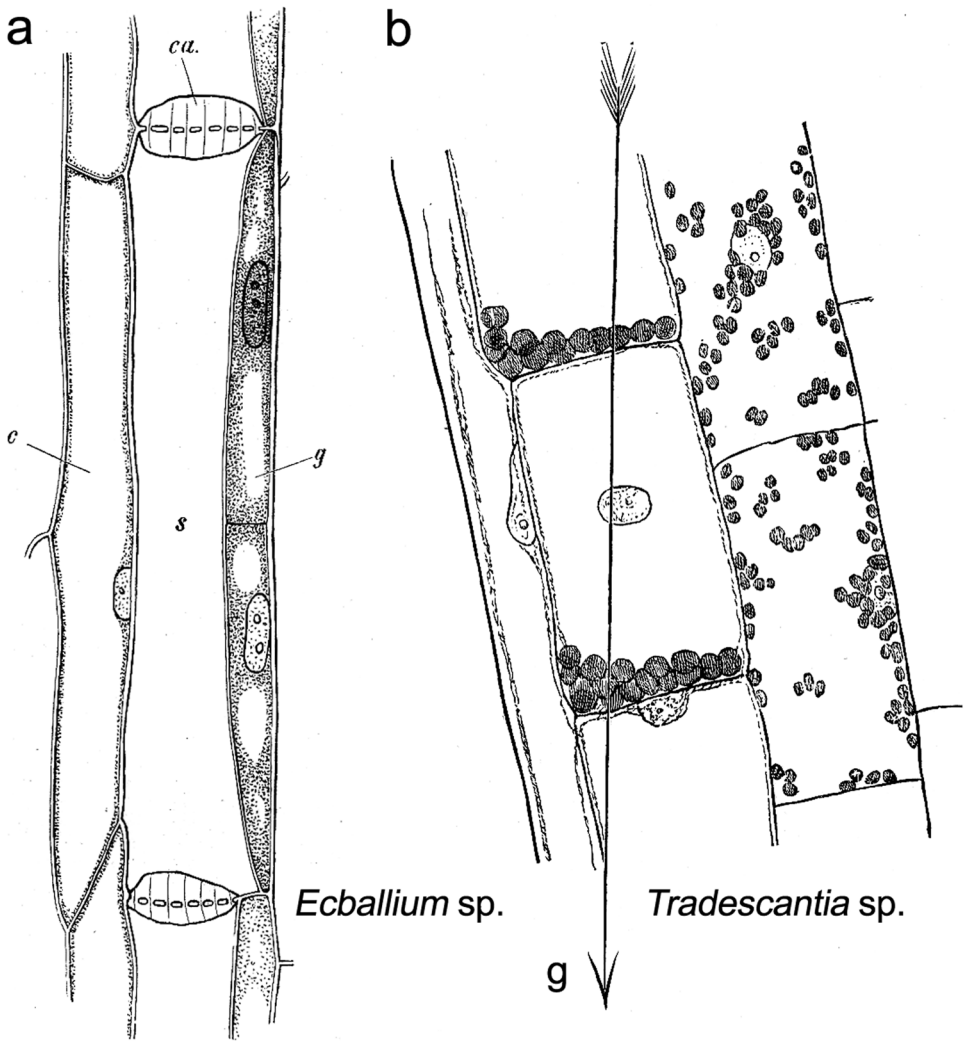

Fig. 3 Drawing of a sieve tube member (**a**) (*s*), with callose-impregnated sieve plates (*ca*), two companion cells (*g*), and a parenchyma cell (*c*) in a stem of *Ecballium elaterium* ("squirting cucumber"). **b** Gravity-perceiving statocytes with gravity-sedimented statoliths (starch-filled amyloplasts) plus adjacent parenchyma cells with ordinary (not sedimented) chloroplasts, in a root of *Tradescantia*. g = gravity vector (from Haberlandt 1924)

Haberlandt's method of gathering information for his *Physiologische Pflanzenanatomie*, which he explained (Haberlandt 1902b), and has sometimes been called "analogy" (Härtel 2003), did not involve experimenting with the functions of different tissues, which would have been far too time- and resource-consuming for the breadth of taxonomic and functional coverage that he aimed at. It was based instead on careful study of the microscopic details of tissues' cell structure, combined with reasoning about function based on the location of a tissue, what function(s) need to be serviced there, and how the tissue's cellular structure physically suits it for performing one, or some, of those functions. One example, the structure of a sieve tube, is shown in Fig. 3a. Importantly, Haberlandt discovered and described the role of statoliths (intracellularly mobile, starch-filled plastids) in cells that are capable of perceiving gravity (statocytes) (Fig. 3b). These studies were the basis of the "starch-statolith-theory" of gravity perception by plants published independently by Němec (1900) and Haberlandt (1900). This insight was presented on March 12, 1903, by Francis Darwin (1848–1925, Charles Darwin's son) in a paper read at a Royal Society meeting, and was subsequently published in *Nature* (Darwin 1903). Today, the Němec-Haberlandt theory of gravity perception is still a matter of debate (see Kutschera 2001 *vs*. Edelmann 2018).

Some important physiological functions, such as active transport through cellular membranes, were not recognized at that time, and/or do not depend physically on overall cell structure, so certain now-important functions were missed. Nevertheless, it is remarkable how close to present-day organismal plant physiology Haberlandt's text in most cases reads. The well-known late-nineteenth-century plant physiologist A. F. W. Schimper (founder of the field of physiological plant geography), who tested experimentally some of Haberlandt's functional deductions, confirmed most of them (Noé 1934). Today, the elasticity and irreversible bending responses of lateral branches of woody plants are one topic of plant biomechanics (Ray and Bret-Harte 2019). As Fig. 4 illustrates, Haberlandt (1924) studied the anatomical

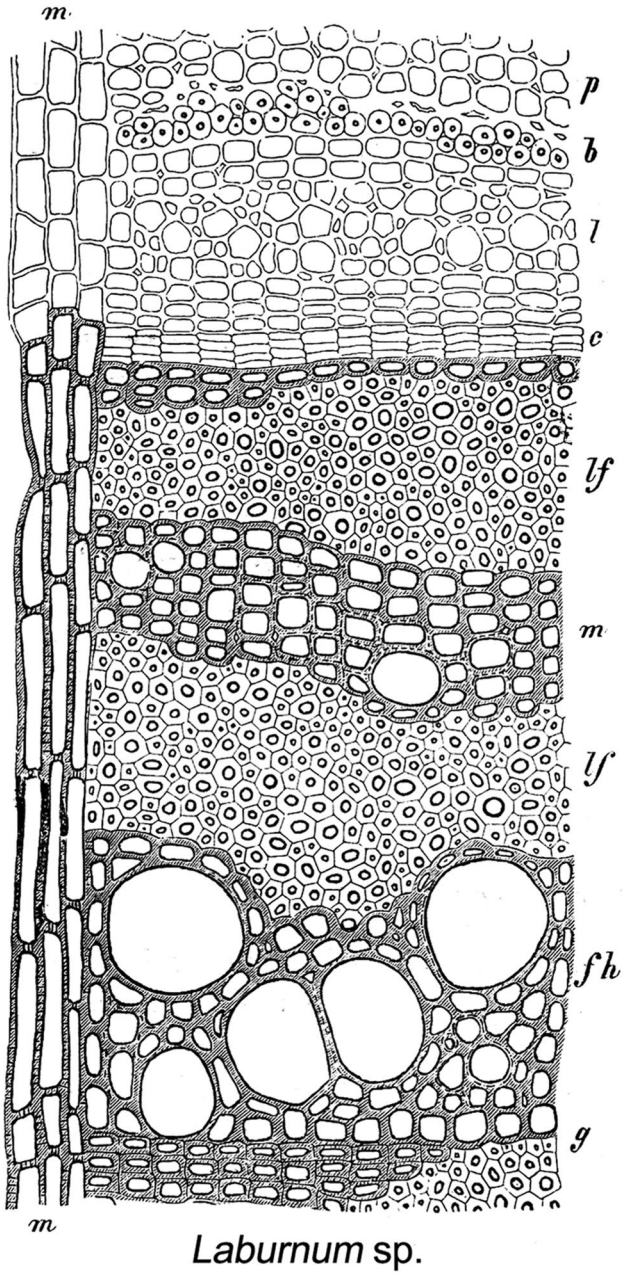

Fig. 4 Anatomy of wood and bark of a 12-year-old branch of *Laburnum anagyroides* (Fabaceae: "golden chain tree") at the end of October. The drawing shows a small part of a cross-section. (*c*), Vascular cambium, below which is xylem (wood), and above which is phloem (inner bark). In the xylem, wood fibers (*lf*), tracheids (*m*), vessels and tracheids (*fh*), wood ray cells (*m* at bottom of drawing), annual ring (*g*), and below it, latewood (tracheids and fibers). In the phloem, sieve tubes and companion cells (*l*), phloem or bast fibers (*b*), and parenchyma (*p*) (from Haberlandt 1924)

features of the wood of tree branches and discussed their mechanical properties.

How much functional–anatomical information Haberlandt added, using his method of investigation, to *Physiologische Pflanzenanatomie* subsequent to its initial publication can be judged from the contents, in pages and illustrations (most of the latter being Haberlandt's drawings), in its successive editions:

First edition 1884: 398 pages/140 figures
Second edition 1896: 550 pages/235 figures
Third edition 1904: 616 pages/264 figures
Fourth edition 1909: 650 pages/291 figures
Fifth edition 1918: 670 pages/295 figures
Sixth edition 1924: 671 pages/295 figures

The first edition of *Physiologische Pflanzenanatomie* stirred up controversy in the botanical teaching world, because Haberlandt substituted, for the then-accepted classification of plant tissues based on their cell morphologies, a classification based on 10 different "physiological systems". This controversy, which was interestingly reviewed by Otto Härtel (2003), persisted for decades, so that although *Physiologische Pflanzenanatomie* became universally acknowledged as an extremely valuable and, with its successive editions, an ever more extensive source of plant-anatomical information, many older botanists would not use it in teaching because Haberlandt retained his classification, to which they objected, in successive editions. The book's value, however, has continued to this day, considerably outlasting that of almost all other plant anatomy books ever published.

Journey to the tropics and cultivation of cells

After clearing initial hurdles with *Physiologische Pflanzenanatomie*, Haberlandt availed himself in 1891–1892 of an opportunity to travel to the tropics, to what are now Indonesia and Malaysia, and work at the botanical garden in Buitenzorg (Java), where he must have obtained much tropical-plant anatomical information to add to future editions of his textbook. He was also able to travel around in the area, to see and make pencil sketches of different kinds of tropical vegetation. Upon returning to Graz, he published a small book, *Eine botanische Tropenreise* (A botanical tropics-journey, Haberlandt 1893) in which 51 of his sketches were reproduced; the book became quite popular with botanists, going through 3 editions.

Later in the 1890s, Haberlandt became interested in how cell growth, cell division, and cell differentiation, which occur during organ development in stems, roots, leaves, and flowers which he had studied, are controlled. He had accepted a basic concept of cell theory, i.e., that cells with their protoplasmic contents are the minimal living units of all organisms (cells are "elementary organisms"; see Brucke 1862 and Haberlandt 1925). With reference to the organismal theory of plant structure, he realized that a way to investigate controlling actions on organ development would be to

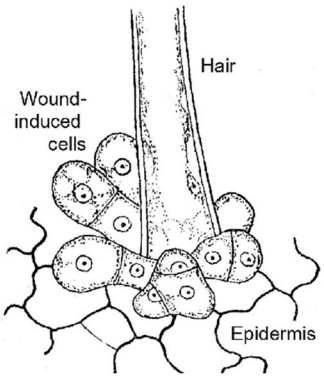

Fig. 5 Effect of wounding, by cutting off the tip portion of an elongated epidermal hair cell, on cell division activity on the upper surface of a leaf of *Pelargonium zonale*. The newly generated chains of isodiametric cells are reminiscent of callus tissue. Ordinary epidermal cells with wavy sidewalls are visible below the callus cells (from Haberlandt 1921a)

grow isolated tissues in culture with the aim of finding out what kinds of other cells, or substances, might make them carry out any of these processes. Thus, in 1898, Haberlandt started trying to create plant tissue cultures (Höxtermann 1997). Although in his preliminary experiments cells sometimes grew in size, none of them divided or differentiated.

Despite these negative results, he evidently felt that the idea of cell or tissue cultures was important to publish, for in 1902, he did so along with information about his results thus far (Haberlandt 1902a). Apparently, this preceded any reports of actual cell or tissue cultures in animal or human biology (Härtel 2003), so Haberlandt can here be credited with the idea of an important, novel approach to cell biology that has blossomed extensively in later years, in both animal and plant research. His (Haberlandt 1902a) paper also mentions the issue of whether any differentiated cells could reverse this and differentiate in some other way, or even grow and divide to yield an entire plant with all its differentiated cell types. Hence, the concept of epigenetic *totipotency*, with which subsequent plant and animal cell culture work has been considerably preoccupied, was founded. This was another important foresight registered in 1902 by Haberlandt.

When Simon Schwendener retired as Professor of General Botany in Berlin in 1909, he had evidently kept up with Haberlandt's doings in Graz and retained his view that Gottlieb had been his most outstanding student, because Schwendender recommended that for his successor Berlin University should choose Haberlandt, which it proceeded to do. Since this professorship was, as mentioned earlier, essentially the most prestigious botanical one in all of German-speaking central Europe, it would have been difficult for Haberlandt to refuse it, so despite leaving beautiful Graz in 1910 "with a heavy heart" (Noé 1934), the honor of the Berlin position prevailed, along with its ability to attract high-quality research students, and the promise given him of a newly-to-be-built institute of plant physiology in the suburb Dahlem. However, although in the fall of 1910 Haberlandt started giving academic lectures in the university buildings in Berlin, it was 3 years before the new institute (complete with greenhouses and gardens) could be occupied. Soon thereafter, World War I started, creating difficulties for everyone, including Haberlandt's absences from the university to serve on war-related services such as a food allocation board.

Plant tissue cultures and the wound hormone

Once Haberlandt was established, post-WWI, in Berlin/Dahlem, graduate students chose to work with him to continue his effort to obtain plant tissue cultures. Although they never obtained indefinitely long-continued cell cultures, partly because for creating and maintaining them, they did not adopt bacteriological sterile technique (with which they may not have been familiar). However, they did discover several situations—including wounding responses, flower fertilization, parthenogenetic (fertilization-less) fruit development, and adventive embryony (de novo embryo development from non-reproductive cells)—in which it seemed clear that a stimulus coming from adjacent tissue or cell(s) was inducing cells to divide. Also, cell totipotency was indicated in the case of adventive embryony.

These findings were written up in student PhD theses and then published in journal papers authored by Haberlandt about 100 years ago (1921a, b). In these articles (see also Haberlandt 1922, 1925), he used the term "hormone," then recently introduced into animal physiology, to denote his proposed cell division–inducing (presumably chemical) stimulus, so Haberlandt can be credited with originating the concept of plant hormones, a priority usually awarded to Boysen-Jensen and Nielsen (1926) and Frits Went (1926) for having detected the transmissible, chemical growth stimulus ("Wuchsstoff") that we now call auxin. Figure 5 shows Haberlandt's illustration of the results of a key experiment, the induction of cell division activity as a result of tissue wounding. It was many years after 1921 that Haberlandt's "cell division stimulus" was actually demonstrated, in plant tissue cultures, to be the adenine derivatives now called *cytokinins*, of which kinetin was the first to be identified (Miller et al. 1955). The classical systems for in vitro plant regeneration, based on excised (sterile) pith sections from tobacco (*Nicotiana tabacum*), are described in textbooks of plant physiology (Ray 1972; Kutschera 2019).

After briefly noting the rest of Haberlandt's life, we proceed below to review the kind of technique that has developed for plant tissue culture and organ or whole plant regeneration, since the time of Haberlandt and his successors in this field, based on the present authors' experience.

Gottlieb Haberlandt's final years, his memoirs, and demise

In 1914, several years after Charlotte Haberlandt's death, he re-married (to Emma Klengenberg). Sometime thereafter, he is said to have suffered a "serious street accident," (encounter(s) with then-novel "automobiles"?) from which Emma's care enabled him to make some recovery (Härtel 2003). One century ago, he started to prepare the 6th edition of *Physiologische Pflanzenanatomie* in time for its publication in 1924 and wrote his memoirs, which were published 9 years later (Haberlandt 1933). He lived on in Berlin into and through most of the Second World War, until January 30, 1945. On that day, Gottlieb Haberlandt died at age 90, shortly before one of that war's most destructive Allied bombing air-raids on Berlin. This raid, which actually occurred as his funeral was being held, completely destroyed his home with all his treasured possessions including books, many years of research notes, and most of his self-made paintings and drawings (Höxtermann 1997). Thankfully, through his death, he escaped experiencing this loss. It should not be forgotten that this kind of war was started several years earlier by Nazi Germany with its terribly destructive, no-holds-barred "Battle of Britain" aerial bombings of many British cities, including London. Ordinary Germans, or Austrians like Haberlandt, would of course have been powerless to stop dictator Adolf Hitler (1889–1945) from ordering these air-raids. However, that the Allies' subsequent aerial retribution was part of what was needed to end WWII and Hitler's aggressive, world-subjugating Naziism, in retrospect is terribly regrettable, while Haberlandt's Berlin home becoming a target of the allied raid was probably completely accidental (an unintended target).

Post-Haberlandt plant tissue culture and regeneration technique

As noted above, Haberlandt (1921a, b) observed that, as a result of a hormone-mediated wound response, callus-like tissues can develop (Fig. 5). As thoroughly reviewed in Laimer and Rücker (2003) and by Thorpe (2007), Krikorian and Berquam (1969), and others, by the 1930s, sterile culture techniques were being used. In France, in 1934 Roger Gautheret (1919–1997) obtained the first genuine plant tissue culture from vascular cambium of *Acer pseudoplatanus* (field maple). Five years later, he, P. Nobécourt, and P. R. White all independently achieved indefinitely proliferating cultures (i.e., cell lines that remained "forever young"). Gautheret continued extensive work in this field and was still around nearly 50 years after 1934 to write a brief but poignant history of the subject (Gautheret 1983). The obligatory role of plant hormones, especially auxin and eventually (1955) cytokinin, in plant tissue culture was recognized, and shoot and root regeneration in cultures was occurring. This was later utilized to make a possible molecular-genetic modification of crop plants, by providing a method for regenerating whole plants from cells whose genomes had been molecularly modified. Still more recently, the concept of *stem cells*, which had been developed in animal work to explain cell differentiation and tissue renewal after injury

or loss, was extended to plants (Sharma and Fletcher 2002; Sablowski 2004, 2010; Greb and Lohmann 2016).

Stem cells have a capacity for indefinitely continued "self-renewal" by mitotic division activity (Fig. 2), combined with an ability (called "potency") to differentiate into specialized cell types. A stem cell capable of differentiating into any of a body's cell types is called *totipotent.* The fertilized egg and its immediate cell-division daughters are the only animal examples. A stem cell that could differentiate into any of the embryo proper's tissues (i.e., into anything except accessory nutritive or positioning structures derived from the egg, like placenta and umbilical cord) is called *pluripotent*. Seed plants have an equivalent of this, because their embryos develop at first an enlarging or elongating cellular structure called the suspensor, which moves its tip, consisting of pluripotent cells that become the actual embryo plant, to a favorable position within the developing seed, and may contribute to the embryo proper's nutrition, but not to its cellular growth.

As growth and development proceeds, the potential of most of the pluripotent cells for future differentiation gets progressively restricted, as displayed by Waddington's concept of a "developmental landscape" (Wang et al. 2011), to a narrower and narrower range of cell types. Such stem cells are called *multipotent*. Before one of these actually differentiates to become a particularly specialized cell type, its fate presumably becomes restricted to just that cell type, so it could be called *unipotent*. Although this term is not usually used in animal systems, it is needed in plants, because a number of plant tissues consist of a single cell type (e.g., collenchyma, sclerenchyma, simple parenchyma, usually pith, and some of those shown in Figs. 4, 5, and 6), all of whose immediate precursor stem cells are probably unipotent.

During embryo development within the seed, post-germination plant stem cells become localized to apical and lateral cell-division zones called *meristems*. Investigators of plant growth became very familiar with meristems long before the term "stem cells" was applied to them. The zone, within a meristem, in which stem cells with particular characteristics are localized can be called a "stem cell niche," a term apparently adapted from ecology. Rather than, or in addition to, specific physical localization, a stem cell niche implies a specific set of stem cell characteristics such as multipotency restricted to certain cell types; or pattern(s) of cell division or of separation of division daughters that will continue dividing from those that are embarking on the path to differentiation; or susceptibility to particular pathways of epigenetic regulation.

Apart from the interest in stem cells because of their importance to biotechnology, and to molecular-level explanations of development, they have also attracted attention regarding applications for creating commercial products, an area that has its own specialized literature (Aggarwal et al. 2020). Here we describe just one relatively recent, non-commercial tissue-culturing and regeneration project (inevitably involving stem cells) with which we happen to have personal experience. This will provide an example of plant tissue culture procedure, the kinds of issues that can be encountered, and some of the conclusions that can be reached from the results. In the discussion thereafter, we comment briefly on the current surge of powerful molecular investigations into shoot and root regeneration in plant tissue cultures, in which the stem cell concept figures prominently and which harkens back to the related concepts due originally to Haberlandt (1921a, b).

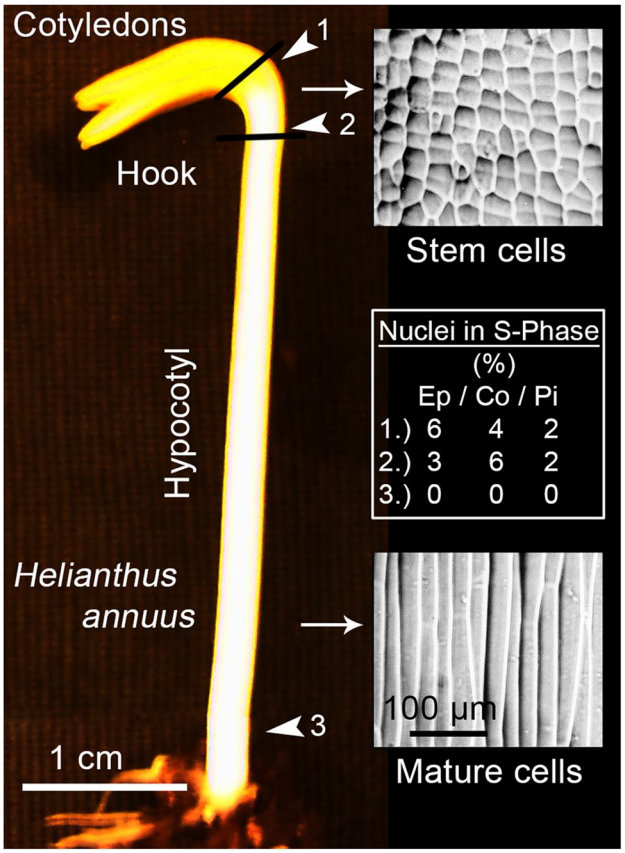

Fig. 6 Photograph of a 4-day-old etiolated sunflower seedling. In the area of the apical hook, which represents a stem cell niche, meristematic tissue can be observed in the light microscope. In the basal region of the organ, mature, fully elongated epidermal cells are visible. Assays of meristematic activity in the epidermis (ep), cortex (c), and pith (p) in locations 1, 2, and 3 are included (data from Heupel and Kutschera 1996). Transverse dark lines at 1 and 2 show locations of cell cycle assays but not of the cuts for hook segment excision, which were as in Fig. 7b

A plant tissue culture/regeneration experiment

Fifteen years ago, Koopmann and Kutschera (2005) published a preliminary report on in vitro regeneration in a

crop plant. They investigated excised segments from sunflower seedlings, incubated on sterile culture media, with respect to their ability to form *callus*, an unorganized mass of dividing cells (e.g., Fig. 5), and to produce new shoots or whole plants. However, in this short article, important details were omitted and no concise interpretation of the results was provided. The following account is based on the original protocols. It describes the exact culture procedure used to obtain miniature intact plants, some of which actually flowered. The specific culture experiment shown here was designed to test whether putative symbiotic prokaryotic microbes called methylobacteria, which occur on or within field-grown sunflower plants, are necessary, or helpful, for plant regeneration. The experiment also calls attention to other issues about regeneration that is discussed below.

Figure 6 shows the experimental dark-grown sunflower (*Helianthus annuus* L.) seedling's hypocotyl (transition zone between root and shoot) starting material, consisting of a straight portion and an apically located "hook," in which part of the hypocotyl's growth in length is occurring. The upper photomicrograph in Fig. 6 shows some of the hook's longitudinal files of short, uniformly sized cells, which must be dividing in order to maintain their small size as the tissue elongates. Hence, they must be meristematic (stem cells), which have been shown biochemically to be dividing (Heupel and Kutschera 1996). In addition, it has been documented that the hook displays the largest metabolic activity of the developing sunflower seedling (Kutschera and Niklas 2011, 2012). This may be due to the fact that the meristem contains many more mitochondria per protoplasmic unit than the more basal cells of the organ. As elongation of this curving hook axis lengthens it, its basal part straightens and adds to the already-straight axis below. As a result, cell division ceases, but elongation continues for a while in the most recently straightened part. The end result, shown in the lower photomicrograph in Fig. 6 of a part of the basal region of the hypocotyl, is that the cells have become greatly elongated. The basal part of the hypocotyl is well below the elongation zone, so these cells must no longer be either growing or dividing. Obviously, they are not stem cells, according to the definition of stem cells as "self-renewing by divisions" (Greb and Lohmann 2016).

Segments cut from these two tissue zones were tested in the following procedure. It used a regeneration culture medium (RgM) nearly identical to that developed, by Paterson and Everett (1985) from earlier procedures and media, to reliably obtain callus and regenerated shoots from sunflower basal hypocotyl segments.

Four-day-old, dark-grown (etiolated) sunflower (*Helianthus annuus* L.) seedlings were raised from sterilized seeds, in glass jars containing sterile vermiculite that was moistened with sterile Murashige and Skoog (1962) mineral salts medium ("MSM"). Under sterile conditions under dim green light, from the hypocotyl hook of each seedling, a segment ca. 4 to 5 mm long was cut such that its basal end was located 2 to 3 mm behind the tip end of the hook, and its apical end embraced about 2 mm forward of that point to include the basal ends (most of their respective petioles) of the two cotyledons (Fig. 6). These are attached to the hypocotyl tip at that point and, located between those 2 basal ends, the not-yet-elongated and not-yet-visible from outside without dissection, epicotyl's apical bud (shoot tip) (cut shown in Fig. 7). In this context, epicotyl refers to the entire, but as yet undeveloped, shoot system above the cotyledonary node. A segment similarly long, but containing no cotyledonary bases nor shoot apical bud, was also cut from the basal (lower, straightened) portion of the hypocotyl as indicated by the lower arrow in Fig. 6. For the first incubation, segments were placed separately on a modified version of the "shoot induction medium" listed by Torres (1989, p. 99): sterilized, agar-solidified MSM that was supplemented with 3% (w/w) sucrose, 0.5% KNO_3, 0.01% myo-inositol, the cytokinin benzyl adenine (BA, 1 mg/L), and gibberellic acid (GA, 0.1 mg/L), i.e., regeneration medium, "RgM." The only significant difference between it and Paterson and Everett's (1985) regeneration medium was the presence of auxin (NAA) in the latter but the absence of any auxin from RgM. Auxin was omitted from the RgM because Paterson (1984) had found that shoot regeneration from sunflower seedling apical buds, which (as explained below) was the source of our shoot regenerants, was inhibited by exogenous auxin, with callus developing instead.

About half of the RgM samples were inoculated with a solution containing methylobacteria (*Methylobacterium* sp.) using a pure culture thereof that had been isolated from a field-grown sunflower plant. The other ca. 50% of the explant cultures (the controls) received an equal volume of sterile water. Cultures were incubated for 14 days in darkness (25 °C), during which callus started to grow at the explants' basal ends, followed by 14 days in a 16:8 h light: dark cycle, which induced regeneration of shoots (Fig. 8a, b). When they became large enough (as in Fig. 8b), shoots were removed from their respective hypocotyl segments and kept for 20 days in darkness in a glass jar with their bases inserted into sterile MSM-agar medium–containing sucrose, GA (2.0 mg/L), and several amino acids (modified from one given on the upper part of Torres 1989 p. 103). They were then inserted basally, for root initiation, into an MSM-agar medium that contained sucrose and the vitamin myo-inositol (as above), plus the synthetic auxin naphthalene acetic acid (NAA, 0.1 mg/L), i.e., rooting medium, "RtM" (Torres 1989, p. 103). Over the following 50 days, plant development was documented photographically (Koopmann and Kutschera 2005).

To check, at the end of the experiment, on the sterility of the control (aseptic) samples and on the success of the

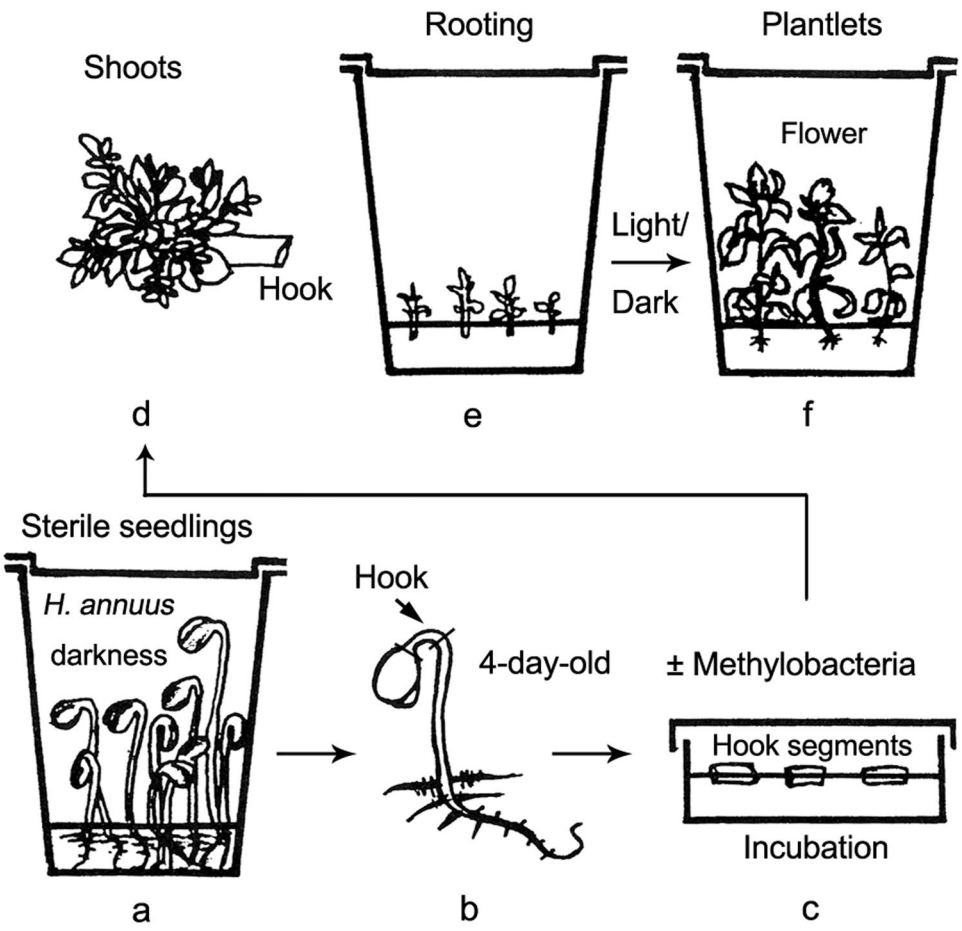

Fig. 7 Procedure for in vitro regeneration using segments cut from the hypocotyl hook of germ-free, etiolated sunflower seedlings as basic material (**a**). Using sterile technique, hook segments were excised between the 2 transverse lines shown in **b**, and incubated (**c**) on a shoot-inducing agar medium (RgM). The regenerated shoots (**d**) were removed from the hook segment, planted temporarily on an amino acid- and GA-supplemented medium, and then on a root-inducing (RtM) auxin-agar medium (**e**). The resulting miniature sunflower plantlets, raised in a light/dark regime, eventually developed flowers (**f**) (see Fig. 8c)

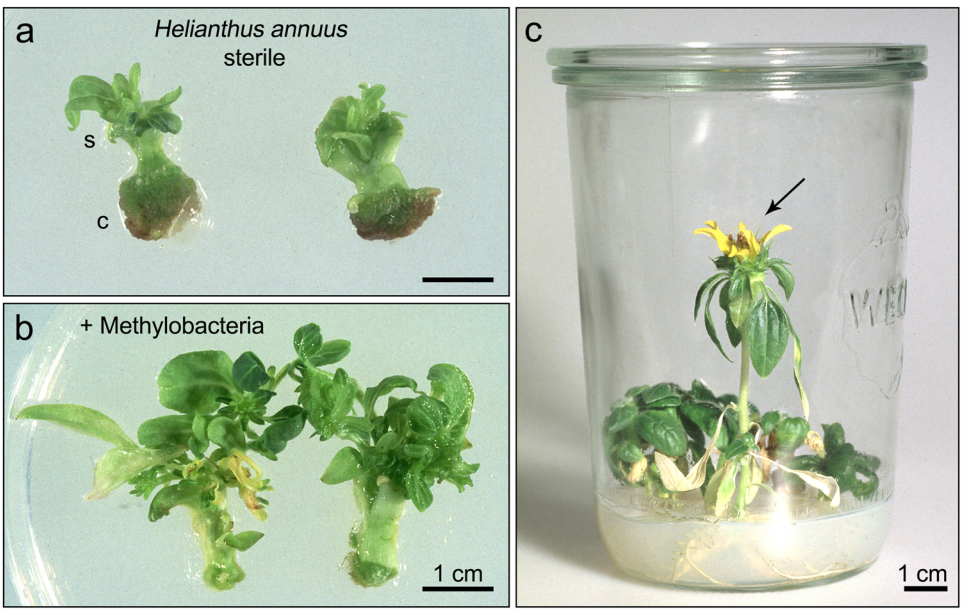

Fig. 8 After incubation on RgM medium in the light in the absence (**a**) and in the presence (**b**) of methylobacteria callus (c) had developed at the basal ends of sunflower hypocotyl hook explants and shoots (s) had regenerated from the epicotyl bud at the tip end of the hypocotyl (located between the bases of the 2 cotyledons, which are visible to either side of the cluster of shoots). **c** After 50 days of growth in a light/dark-cycle under sterile conditions, miniature flowering plantlets had developed morphological patterns similar to field-grown (non-sterile) sunflowers (arrow)

performed methylobacterial inoculations, pieces excised from the leaves of 50-day-old regenerated plants (Fig. 8c) were placed on sterile nutrient-agar plates. One day later, these pieces were removed and the plates were incubated for a further 7 days in darkness.

Results and interpretation

The just-mentioned terminal microbial culturing tests on the controls gave no bacterial or fungal growth, whereas the inoculated samples yielded heavy pink colonies of methylobacteria, as expected (Fig. 9a). Scanning electron micrographs of the epidermal surfaces of similar leaf pieces (Fig. 9b) showed the presence of rod-shaped methylobacterial cells in the inoculated samples but not in the controls.

Figures 8a and b show representative sunflower hook explants with outgrowing regenerated shoots, after 28 days of incubation on RgM. The lump at the basal end of each explant is callus. The regenerated shoots arise not from the callus, but from the epicotyl's apical bud located between the two cotyledons' bases, which have been spread apart by the mass of young shoots that has grown out between them. Similar shoot clusters arose from the epicotyl apices of sunflower seedlings when Paterson (1984) dissected them out, cut them in half lengthwise, and cultured them on an agar medium. Our regenerated shoots appear to have the same origin, but from a still-attached (to the explant) apical bud rather than an isolated halved one.

Noticeably more regenerated shoots (8 ± 2 per explant) arose in the methylobacteria-inoculated samples than in the controls (5 ± 1) ($n = 18$). As can be seen in Fig. 8, the inoculated-sample shoots were also noticeably larger than in the controls, and the amounts of callus were correspondingly smaller. Upon incubation on RtM, more roots were initiated in the inoculated samples (10 ± 3 per shoot) than in the controls (7 ± 1) ($n = 18$). Judging from the sizes of the standard errors, all these differences are statistically significant. In addition to increasing the induced root numbers, inoculation also increased the percent of shoots that rooted, from 61 (controls) to 92% (inoculated) ($n = 100$). However, the shoot growth difference was seen in Fig. 8a, b proved to be transient, the mean stem lengths of the control and inoculated shoots in culture eventually becoming indistinguishable (respectively 22 ± 1 and 23 ± 1 mm after 20 days, or 30 ± 3 and 28 ± 4 mm after 45 da) ($n = 100$).

The results show that neither methylobacteria nor other microorganisms are required for the regeneration of sunflower plantlets from apical hypocotyl hook segments.

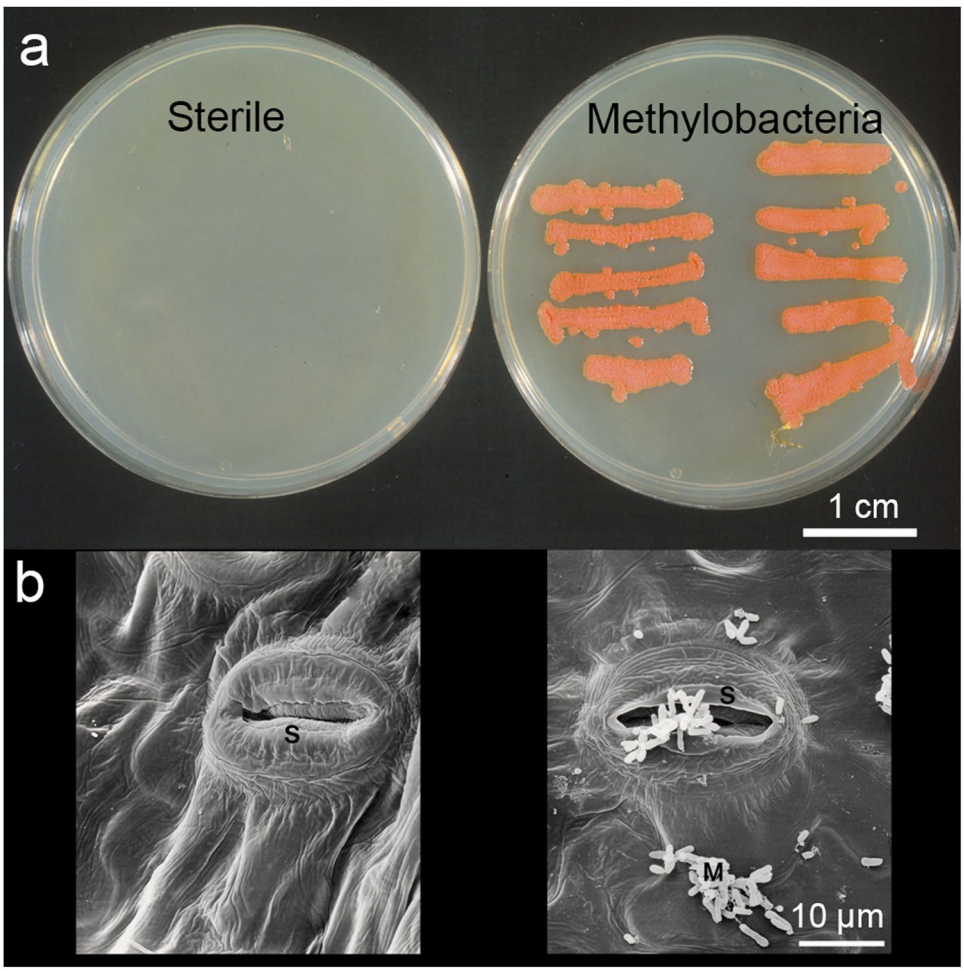

Fig. 9 **a** Experimental verification that, in plantlets like that shown in Fig. 8c, ligules taken from an aseptically raised flower were sterile (left sample), while those from an inoculated culture contained pink methylobacteria (right sample). **b** On the green leaves of aseptic 50-day-old sunflower plantlets, no epiphytic bacteria were observed using scanning electron microscopy (left sample), whereas on the plantlets raised with methylobacteria, numerous microbes were detected (right sample). Methylobacteria (M) formed clusters on the cuticle of the epidermal cells that, in several documented cases, also occurred around the stomatal pore (s)

Possible reasons for the promotion of shoot regeneration and root initiation by methylobacteria are considered below.

Figure 8c shows that even in the absence of methylobacteria, i.e., in sterile plantlets in culture, flower heads developed that were miniatures of the species-typical composite heads of field-grown *Helianthus annuus*. We can conclude that bacteria, symbiotic or not, are unnecessary for flower differentiation and development. It would have been interesting to test whether these regenerated, flowering *H. annuus* plantlets would display this species' natural solar-tracking response (Kutschera and Briggs 2016), but we unfortunately did not determine this. In addition, whether or not these plantlets develop lateral roots in response to sucrose, as documented for sunflower seedlings (Kutschera and Briggs 2019), is unknown.

The same procedure (Fig. 7) was performed using hypocotyl segments cut from near the base of the straight hypocotyl axis (lower arrow in Fig. 6). These organ fragments, composed of mature, very elongated cells (Fig. 6) that were neither growing nor dividing, did not develop significant callus, and in none of the samples was shoot regeneration observed. Inoculation of the cultures with methylobacteria did not alter these results (unpublished observations).

The striking feature of the presently described results is that our subapical hook segments, on a medium closely similar to Paterson and Everett's (1985) but without auxin, regenerated both callus and new shoots extensively (Fig. 8). Whereas basal segments like those they worked with would not do so unless (as Paterson and Everett 1985 found) exogenous auxin was supplied to them.

Effect of inoculation with methylobacteria

An obvious possible basis for our observed stimulation of shoot and root regeneration by methylobacteria (Fig. 8) is that some strains of these bacteria are known to produce cytokinin (*trans*-zeatin: Lidstrom and Christoserdova 2002; Klikno and Kutschera 2017; Krug et al. 2020) and/or auxin (IAA: Hornschuh et al. 2006). Since our RgM contained no auxin, it seems possible that bacterially-produced auxin and/or cytokinin could have increased the number of initiated shoots and roots, as well as shoot growth shortly after initiation. However, we do not know whether the sunflower-derived methylobacterial strain used in this experiment actually produces auxin or cytokinin, so an alternative hypothesis needs consideration.

Plants produce and release methanol (Fall and Benson 1996). It may arise from the methyl ester groups of cell wall pectins, which the pectin methylesterase, that cell walls can also contain (Bosch et al. 2005), will hydrolyze to release MeOH. There may also be intracellular sources of MeOH in side reactions from methylation processes.

Methylobacteria can use methanol as a nutrient (Krug et al. 2020). It might, therefore, be a chemotactic attractant for strains of methylobacteria that are motile (Schauer and Kutschera 2011; Doerges and Kutschera 2014). This might explain the observed aggregation of methylobacterial cells in, and outside of, stomatal apertures (Fig. 9b), which are sites of MeOH emission from plants (Abanda-Nkpwatt et al. 2006).

The level of methanol that is being produced within the regenerating hypocotyl hook tissues might be somewhat toxic to them (as MeOH ["wood alcohol"] notoriously is to humans), and thus somewhat inhibitory to regeneration. Consumption of methanol by methylobacteria would reduce the tissue-internal MeOH level (Abanda-Nkpwatt et al. 2006) and thereby reduce or eliminate its inhibition of regeneration, which would thus be stimulated. But methylobacterial inoculation has not stimulated normal (but otherwise germ-free) sunflower shoot or root growth (Kutschera 2007). This might just mean, though, that either normal plants' internal methanol level, or the sensitivity of normal tissue's growth to methanol, or both, are lower than in the cultured explants so the normal organs' growth is not being partially inhibited by MeOH, and hence cannot be stimulated by its removal by bacterial consumption. Why inoculation reduces callus growth (Fig. 8b vs. Figure 8a) might be because inoculation-stimulated shoot growth during incubation competes with callus for resources that they both need for growth.

Source of auxin for regeneration

Our experiment indicates that some of the hypocotyl hook region's stem cells are capable, in the absence of exogenous auxin, of producing callus and regenerate shoots. The same tissue just a few days older (then located basally, in the portion of the hypocotyl hook that has previously straightened), which is no longer growing nor meristematic, and thus apparently no longer contains stem cells, will perform these regenerations only if an auxin, such as NAA, is supplied to them exogenously (Paterson and Everett 1985). The weight of evidence from the shoot regeneration literature is that auxin is required for both this regeneration and for callus, the former taking place only after, and indeed within, the latter after its auxin-induced development. This was indeed the picture presented by Paterson and Everett's (1985) induction of callus and shoot regeneration in basal sunflower hypocotyl segments. Literature discussed below supports the involvement of auxin-dependent steps not only in callus development but also in shoot apex initiation within the callus. Therefore, our results suggest that sunflower hypocotyl hook explants contain an endogenous auxin source(s) that satisfied the physiological needs of both callus growth and shoot regeneration.

The meristematic tissues of the apical region or bud of a shoot are the principal sites of auxin production in a seed plant, so the epicotyl apical bud included in our hook explants should have been an auxin source. The hypocotyl hook portion of our explants is also meristematic (Fig. 6), so auxin is likely being produced within its tissue, as was inferred from molecular data on *Arabidopsis* hypocotyl hooks (Abbas et al. 2013; Zádníková et al. 2010). Auxin from these sources would be moved basipetally by the auxin polar transport system and would accumulate at or near the cut basal end of the explant. Since callus requires an elevated auxin level for initiation, as suggested by Paterson's (1984) earlier-mentioned finding with auxin treatment of sunflower epicotyl buds, the expected basal accumulation would stimulate callus tissue growth at the basal ends of our explants, as occurred (Figs. 7 and 8). Auxin production within the apical bud probably supplied what was needed for shoot regeneration within that bud, as it evidently did for Paterson's (1984) halved apical buds. Auxin production in hook tissue alone might be great enough to induce, in isolated pure hook segments lacking the epicotyl's apical bud, both callus development and new shoot initiation in the absence of exogenous auxin, but that test unfortunately was not included in the present experiment.

Shoot regeneration from the epicotyl's apical bud

New shoot formation from our hypocotyl-hook explants differed importantly from Paterson and Everett's (1985) shoot regeneration from basal sunflower hypocotyl explants, in that the present shoots originated, not in basally induced callus as theirs did, but (as mentioned above) as clusters of about 5 to 8 or more shoots from the epicotyl's apical bud, where no callus developed. The new shoots, like those Paterson (1984) obtained by culturing halved apical buds, almost certainly arose from pre-existing meristems within the bud, i.e., its apical meristem as well as axillary meristems and the rib meristem that normally occurs below a shoot apical meristem. New shoot apical meristems that developed in any of these meristematic areas could probably grow out as shoots without having passed through early stages of shoot regeneration from mature tissue such as callus formation and acquisition of pluripotency (Shin et al. 2020). Meristem cells within the epicotyl apical bud presumably already possess pluripotency, or at least multipotency, since during normal growth they later give rise directly or indirectly to all the cell types found within the future shoot system.

The outgrowth of multiple shoots from hypocotyl explants' epicotyl bud (Fig. 8a, b) differs substantially from the single, unbranched epicotyl shoot that would have grown out, above the cotyledonary node, from the epicotyl's apical bud if the seedlings had continued their normal growth. Even if one of the in vitro shoots were traceable back to the seedling's epicotyl bud apical meristem, all the rest of them would probably be traceable to the aforementioned other meristematic (and therefore stem cell) parts of the epicotyl apical bud. These shoots thus represent many cells in those parts of the epicotyl bud being shifted into developmental pathways and endpoints different from their normal developmental fate. It seems fitting, therefore, to recognize this as a kind of shoot regeneration that differs, at least in its initiation, and probably also in some later stages, from shoot regeneration from mature tissue, via callus. That has been the main subject of research on shoot regeneration up to now at the molecular level, as discussed briefly below.

Paterson and Everett (1985) reported that many sunflower genotypes did not regenerate shoots from NAA-treated basal hypocotyl explants like those from the inbred line (SS415B) that they used in most of their experiments. The present, alternative regeneration method might provide a way around genotypic regeneration difficulties that may be encountered if the Paterson and Everett (1985) procedure is applied widely to other sunflower genotypes. However, our method may not be a single-cell route to regenerants (as often desired in biotechnology), since they evidently stem from multicellular meristems, whereas Paterson and Everett's (1985) regenerants arose as embryoids (having forms similar to the developmental stages of sexually produced embryos), so probably had a single-cell origin. Rather than being pluripotent, since these embryoids each developed a suspensor (see Paterson and Everett's Figs. 3 and 4), their initial (callus) cells must have been totipotent.

Recent work on shoot regeneration

In a review of molecular work on shoot regeneration, using mainly *Arabidopsis* and almost all of it reported in publications dated after 2000, Shin et al. (2020) grouped the participating genes and regulatory signals that have been recognized into four temporal stages, involving successively: (1) callus formation with the acquisition of stem cell pluripotency; (2) formation of a shoot promeristem (meristem precursor) within the callus; (3) its development into a "confined shoot progenitor" whose apical meristem, still within the callus, begins to form leaf primordia; and (4) outgrowth of the newly formed leafy shoot. A comparably detailed and more widely aimed (at diverse species and types of regeneration) recent review by Ikeuchi et al. (2019) recognizes essentially the same sequence, as well as departures from it in some kinds of regeneration. Stage 1 seems approximately equivalent to the entirety of what Ikeuchi et al.'s (2017) time-course transcriptome analysis of wound callus initiation covered, detecting changes (mostly increases) in the transcription of about 14,000 genes over the first 24 h after wounding! Among many other things, these reviews

(Ikeuchi et al. 2018, 2019; Shin et al. 2020) deal with the molecular basis for the development of pluripotency, equivalent to what earlier authors would have called totipotency, with Ikeuchi et al. (2019) using the latter term in one place in their Introduction. Near the end of their chapter, they refer to the "exciting….first molecular insight into this enigmatic concept."

Shin et al. (2020) postulated the involvement of no less than 6 classes of plant hormones (cytokinins, auxins, gibberellins, ethylene, abscisic acid, and brassinosteroids) in the shoot regeneration process. Ikeuchi et al. (2017) found that a 7th hormone, jasmonate, is rapidly induced and apparently active in wound callus development, but in their 2019 review of shoot regeneration, only auxin and cytokinin figure prominently, and in multiple ways. Of Shin et al.'s (2020) hormone list, the first 3 are commonly provided in regeneration media, but only the first two seem to be widely essential exogenously for callus development and shoot regeneration. It has long been known (e.g., Dietz et al. 1990) that exogenous auxin rapidly promotes the expression of numerous genes, some of which are likely involved in these regeneration processes, whereas most of the remaining hormones' roles are apparently fulfilled by endogenous production stimulated by the first two and/or by epigenetic changes that some of the first two's direct products induce. Gibberellic acid (GA) seems intermediate, exogenous GA not being usually required, but at least in some cases (e.g., sunflower, Paterson and Everett 1985), GA was reported to "increase the amount of regeneration" (probably the number of shoots obtained).

A curious feature of shoot regeneration reported by both of the above molecular review authors is that its early stages can involve genes and cell-morphological displays that are related to root apical meristems and to lateral root initiation. Certain gene actions later correct this, suppressing the root-like identity features and thus apparently pushing further development toward the contemporary seed-plant shoot's siphonostelic anatomical model. This is interesting because the protostelic vascular anatomy of contemporary seed plants' *roots* resembles the *stem* anatomy of the primitive, spore-reproducing, root-less early vascular land plant shoots which, apart from contemporary *Psilotum* and *Tmesipteris*, are known only from Paleozoic (mostly Devonian) fossils (Kenrick and Crane 1997). Land plants somewhat later in the Paleozoic evolved roots, probably by modification of their underground stems, retaining in these roots the protostelic anatomy of their precursor stems. The stem itself, contrariwise, evolved in the direction of modern stem anatomy, but apparently retained, as evidenced during regeneration, some of its earlier root-like genetic and epigenetic developmental basis. Thus, shoot regeneration may hint at the once-popular notion of ontogeny recapitulating phylogeny, which dates back to the influential nineteenth-century biologist and Haberlandt contemporary, Ernst Haeckel (1834–1919) (see Barnes 2014, and Niklas et al. 2016).

We think that the flow-sheet causal sequence diagrams of gene expressions, actions, and interactions that emerge from the recent molecular work on callus growth and shoot regeneration, and the identification of hormones involved in these processes (Shin et al. 2020), could Gottlieb Haberlandt return to see them, would astonish him beyond all belief. After his years of experimental preoccupation from about 1900 onward trying to establish that a plant hormone that controls plant cell division must exist, and that totipotency (now pluripotency) can be encountered, or elicited, in vegetative plant cells, he would probably be surprised that most of his ideas were corroborated one century later (Shin et al. 2020). But as both Ikeuchi et al. (2017, 2018, 2019) and Shin et al. (2020) point out, much more research (e.g., Ikeuchi et al. 2018) will still be needed before we have a fully adequate understanding of the regulatory circuits that orchestrate these many genes' spatial and temporal expression patterns to create the causal pathways in regeneration that are currently coming into view. Haberlandt (1921a, b), however, would probably at least have been pleased to learn that plants use, both in normal growth and for regeneration, what we now call stem cells (Stammzellen), which are "ewig jung" (Forever Young)!

Conclusions and outlook

The aim of this review was to recount the life and scientific work of Gottlieb Haberlandt (Fig. 1) who, one century ago, described the wounding-induced formation of callus tissue on plant surfaces such as leaves of *Pelargonium* (Fig. 5). This finding, combined with our current knowledge about stem cells (which Haberlandt would simply have called dividing or meristem cells) and of in vitro plant regeneration (which Haberlandt looked forward to obtaining, but never achieved), made possible the research activity called "plant biotechnology." In his history of this discipline, Vasil (2008) pointed out that, 12 years ago, "biotech foods" had been consumed by more than one billion humans. To the best of our knowledge, no harm to the health of these consumers has emerged, so that, despite ideological resistance, it is fair to conclude that "biotech crops" are safe. This remarkable development with positive impacts on human nutrition and environmental health is based on the totipotency (now termed pluripotency) of cultivated plant cells (Figs. 5, 6, 7, 8, and 9), which is needed in order to achieve regeneration of seed-producing, intact plants from cells whose genome has been altered by molecular techniques. As pointed out by Greb and Lohmann (2016), in trees that can survive very long, plant stem cells "remain active over hundreds or even thousands of years." Combined with the principle of plant

regeneration, these specialized cells remain "forever young." The molecular mechanisms responsible for their doing so are now very much under investigation (Ikeuchi et al. 2018, 2019; Shin et al. 2020).

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