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PLANT BIOPRINTING: NOVEL PERSPECTIVE FOR PLANT BIOTECHNOLOGY

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Abstract: Bioprinting is a technical innovation that has revolutionized tissue engineering. Using conventional printer cartridges filled with cells as well as a suitable scaffold, major advances have been made in the biomedical field, and it is now possible to print skin, bones, blood vessels, and even organs. Unlike animal systems, the application of bioprinting in simple plant tissue cells is still in a nascent phase and has yet to be studied. One major advantage of plants is that all living parts are reprogrammable in the form of totipotent cells. Plant bioprinting may improve scientists'understanding of plant shape and morphogenesis, and could serve for the mass production of desired tissues or plants, or even the production of plant-based biomaterial for industrial uses. This perspectives paper explores these possibilities using knowledge on what is known about bioprinting in other biosystems.

Keywords: biomaterial, bioprinting, plant biotechnology, micropropagation, tissue engineering

Introduction: historical framework and basic bioprinting concepts

The concept of bioprinting emerged in the early 2000s. The patent for bioprinting using a common inkjet printer was filed in the US in 2003 and granted in 2006 to Dr. Thomas Boland at Clemson University [DOYLE, 2014]. Since then, studies in the fields of engineering, material science, cell biology, and regenerative medicine have assessed the impact of bioprinting, with the greatest impact being on biomedical science.

The earliest bioprinter used protein and endothelial cells placed in an inkjet cartridge for 2D printing. To create protein, four cartridges consisting of biotin, streptavidin, biotinylated bovine serum albumin (BSA) or biotin-BSA, and only BSA were used to create a pattern in the shape of the word "Biotin" in Times New Roman font size 8. Using the same biopritnter, trypsinized bovine aortal endothelial cells and smooth muscle cells (i.e., cells that had dettached from each other after bonding proteins broke)were suspended in modified Eagle's [EAGLE, 1955] medium (MEM) and 10% fetal bovine serum with a cell concentration of 1×10^5 cells/ml. The cells were printed in a reconstituted basement membrane gel with 3 mg/ml collagen gel. After printing, the resulting single layer of cells was incubated at 37 °C in a CO_2 environment for 30 min to maintain pH before adding liquid medium. Cells were visualized after 72 hunder an epifluorescent microscope revealing that 75% of the mass of printed cells survived [WILSON JR. & BOLAND, 2003]. Separately, 3D printing technology allows the creation of a 3D biological shape by using cells and a scaffold of desirable shape. This technology covers the limitation of 2D printing that is useful only for a surface area instead of a 3D solid object. In the field of 3D

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bioprinting, the bovine aortal endothelial cells is used for 3D bioprinting [BOLAND & al. 2003] to form cell aggregates in layered thin gel alignments.

3D bioprinting in animal cells using an arranged aggregation principle (i.e., the organized alignment of cells, like pixels, in an orderly fashion, similar to printed letters) as a key protocol for tissue engineering and organogenesis. In biomedics, bioprinting can be used for skin grafting by applying skin tissues [LEE & al. 2013] thus playing a significant role in plastic surgery or wound healing. Organ transplants may be possible by 3D bioprinting that woud allow for the growth of a fully functional and fully developed organ [MIRONOV & al. 2011]. Other uses of 3D printing might be in the production of meat *in vitro* from stem cell tissue and lab-grown *in-vitro* meat may contain designated target nutrients and adjustable shape for aesthetic purposes [POST, 2012; MATTICK & ALLENBY, 2013].

Other than bioprinting involving animal cells and tissues, the application of bioprinting to other complex multicellular organisms, especially plants, has not yet been tested or studied. Unlike its direct function in biomedical sciences, plants might be considered less interesting as a bioprinting subject, also because tissue culture and micropropagation already provide a suitable and robust system for producing plant cells, tissues or organs in a sterile *in vitro* environment. Plant bioprinting may be difficult due to rigid plant cell walls, unlike animal cells that do not have a cell wall, although plant cells have a distinct advantage, totipotency, which allows a plant cell, under strict environmental conditions, to develop a tissue scaffold that serves as the precursor for an organ, and then the whole plant itself, organogenic steps that are under strict genetic control. A second possible problem might be the efficiency of cell and tissue regeneration once a scaffold has been printed. In principle, bioprinting would be required to shape plant cells into a scaffold, which would serve as a building block for engineering plant tissues for partial organogenesis to produce specific products rather than a whole plant. Where necessary, the printer cartridge could overlay different building blocks of different cellular origins onto media or substrates containing different inducers such as plant growth regulators (PGRs). This concept is explored in a bit more detail later.

This paper is the first ever proposal for the theoretic possibility of using 2D and 3D bioptinting in plants by relying on earlier successful cases of animal bioprinting and on a rich literature of basic concepts of plant cell, tissue and organ culture.

Plant bioprinting: basic requirements

1. Cells and tissues for *in vitro* culture

A plant cell or tissue can be made to survive, grow and develop artificially *in vitro* when placed on a suitable medium that contains macro- and micronutrients, carbohydrates, vitamins, and PGRs. The most commonly used basal medium is Murashige and Skoog (MS) [MURASHIGE & SKOOG, 1962]. To obtain plant cell aggregates that could form multiple cell clusters and eventually a tissue, cell suspension cultures in liquid media might serve as the optimal printing form when placed in the printer cartridge rather than the use of "dry" cells, which can die easily due to oxidation.

2. Growth scaffold as a template for shaping the product: importance of basal medium, medium additives and plant growth regulators

A scaffold is essential in organic tissue printing as a base to direct tissue growth. Gel material for plant tissue can be calcium alginate, agar, agarose, polyacrylamide, gelatin, or even synthetic material like polyurethane [NEUMANN & al. 2009]. Other cheaper soft material like starch from various source like sago, cassava, and corn can also be used

[HENDERSON & KINNERSLEY, 1998; NAIK & SARKAR, 2001; DABAI & MUHAMMAD, 2005; PUROHIT & al. 2011]. In plant bioprinting, a scaffold would be used as a mold to shape cells so that they will be aligned prior to differentiation using induction by PGRs.

The proposed printing process can take place in two ways: 2D printing or 3D printing. For 2D printing or monolayer printing, cells are simply directed to spread over a 2D area of nutrient gel scaffold before they are left to grow when placed under optimized conditions (Fig. 1). On the other hand for multilayer or 3D printing, a gel is added gradually to adjust the surface before placing another layer of cells or to enclose the surface. In some cases, a gel may also contain a specific concentration of PGRs to match the desirable level that would result in a product (tissue (e.g., parenchyma, sclerenchyma, etc.) or organ (leaf, stem, petiole, stigma, tuber, bulb, etc.). Thus, more than one gel cartridge may be possible, or necessary. 3D or multilayer printing is expected to be followed by directed organogenesis and differentiation in response to PGRs or other optimal conditions (Fig. 2), either optimized *a priori* using assays, or following the published literature. The difference between traditional plant tissue culture (PTC) and the use of a bioprinter will lie in the automation and the precision associated with it.

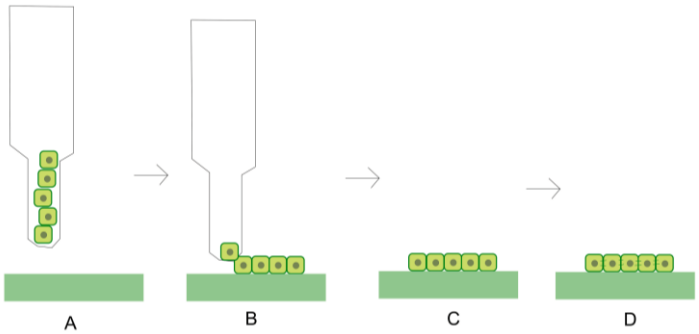


Fig. 1. Monolayer plant cell printing (i.e., 2D) using a simple inkjet printer. From the cartridge nozzle (A), cells are placed over a basal medium (B-C). Some time after, the cells interact and begin a process of differentiation or dedifferentiation (D). Even with a small surface area, bioprinting a layer of cells would take no more than a few seconds to achieve, similar to a regular printer that prints on paper.

When cells are printed, a bioprinter will align each cell to form a layer of equal size forming the desired scaffold. When a 2D printer is used, the printing result will only be a single thin layer of cells of variable sizes depending on the size and capacity of the bioprinter. On the other hand, a 3D printer will add more layers of cells joined by a gelling agent to a desirable height that is limited to a printer's maximum height capacity. A larger printer would thus be able to print alarger scaffold (3D) or wider base (2D). A printed cell or layer of cells (2D horizontal scaffold; Fig. 3A) or cellular mass (3D scaffold; Fig. 3B) may be placed on a basal medium carrying a gradient of PGRs or any other nutrient. The concept of a gradient is not usual in conventional PTC, and is made by using tilted agar medium (Fig. 3A) to make two different concentration gradients that would theoreticallyaffect the growth of the cell layer overlaying it by diffusion. In a 3D gradient (Fig. 3B), the precision of a computer that guides the printing process is important and a step that is impossible to achieve at present in conventional PTC. This gradient medium

serves as an attempt to try and direct the growth of cells that have been printed. The basal medium can either be printed, or is not printed, i.e., it is set *a priori*, e.g., in Petri dishes.

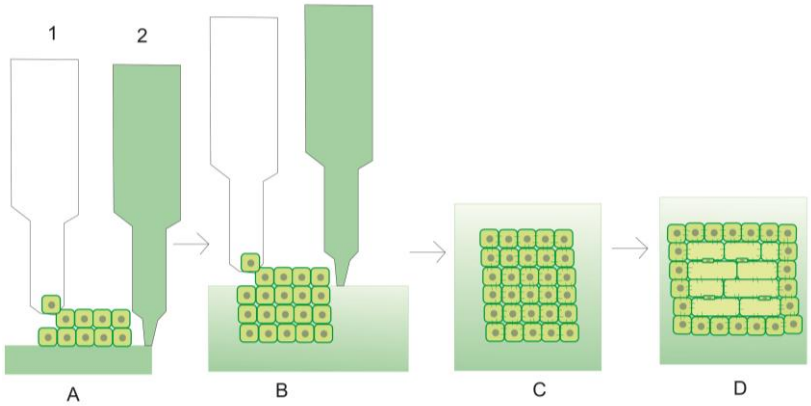
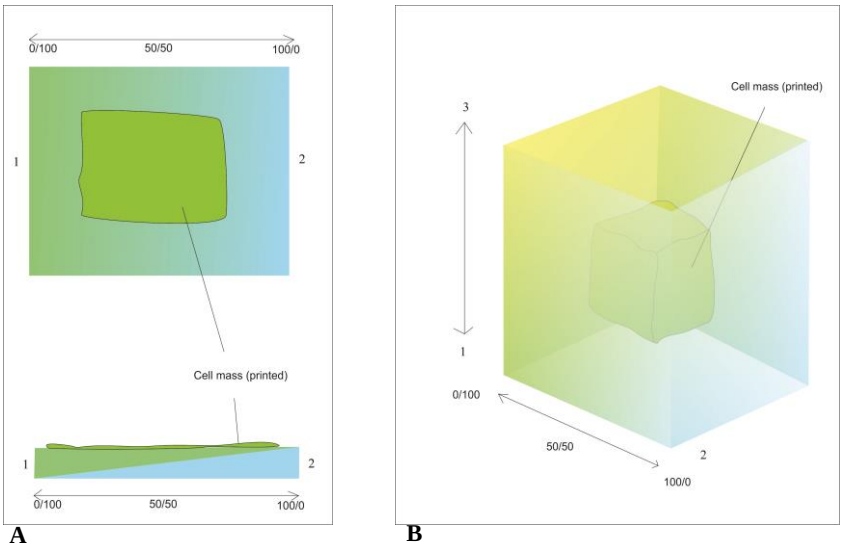


Fig. 2. Multilayer (3D) plant cell printing in which the speed and other specifications can be adjusted. Other than the cell-containing cartridge (1), a gel scaffold with plant growth regulators (PGRs) is placed in another cartridge (2). After one layer is printed (A), the gel is adjusted to add a layer of different cells or a different scaffold (B) to follow the surface of printed cells. Once printing is complete (i.e., resulting in a desired pattern, scaffold or basal structure (C), the interaction between cells, nutrients and PGRs in a basal medium induces the differentiation of cells to form an undifferentiated cellular mass(i.e., callus)or a differentiated mass(i.e., tissue or organ like a leaf or bulb) with a distinct epidermis (D).Depending on the complexity and on the number of different cell types and scaffolds used, which would require different cartridges to be inserted, printing could last from between minutes to a few hours.



A **B**

Fig. 3. Gradient in 2D printed cells (A) showing the ratio between PGR1 and 2.Gradient in 3D printed cells (B). Instead of only two PGRs (1 and 2), the third one (3) can also be added. The intention is to manipulate cellular differentiation for later stages of growth so that a plant product with a desirable shape can be created.

Plant bioprinting: potential applications and perspectives

Bioprinting plant material has foreseeable benefits for ornamental and agricultural purposes, and for biomaterial production (Fig. 4). The main concept in bioprinting using plant cells is to arrange cells into a suitable scaffold of a specified area (2D) or volume (3D)that will allow them to develop, in response to an ideal basal medium and additives, including PGRs, directly into a specific organ (i.e., direct organogenesis).By being able to bioprint a living structure of living cells with desirable shape, the most obvious manipulation that could be envisioned is in the improvement of aesthetic in ornamental plants such as bonsai or *in vitro* flowers (Fig. 4A).

It is also conceivable that plant tissue can be printed as a base for the production of plant-based biomaterial, e.g., lab-grown wood planks or wood blocks for construction (Fig. 4B), decreasing deforestation and creating bioprinted blocks of wood of rare and valuable wood such as sandalwood. Such wood blocks could be printed by tabletop-sized or larger printers.

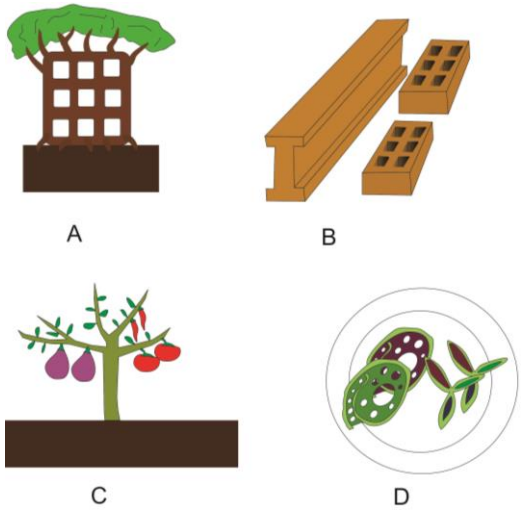


Fig. 4. Applications of plant bioprinting for ornamental plants (A), printed plant-based biomaterial (B), chimeric grafting for horticultural plants (C), and printed plant-based food (D).

A handheld bioprinter could be used for *in-vivo* bioprinting of *in vitro* tissues or onto plants growing under a sterile and controlled environment. For example, a small graft (as a single layer using a 2D printer, or a mass of cells or tissues using a 3D printer) could be printed onto the part of a plant that was damaged by an abiotic stress (e.g., cold- or heat-induced injury) or by a biotic stress (e.g., a fungus or pest) allowing for recovery of dead tissue or covering and strengthening scarred tissue (Fig. 5). As result, chimeric plants that yield multiple fruits can be created (Fig. 4C).

A fourth possibility is the use of plant bioprinting to produce designer plant-based food that combines aesthetics, nutraceuticals, and productional aspects, e.g., lab-grown vegetable products, a procedure equivalent to lab-grown meat. This 3D method would create a desirable and edible plant-based product that can be specific (e.g., only a leaf, root or fruit; Fig. 4D) or a whole printed plant. The procedure would also apply to transgenic material. Such a bioprinter would allow individuals to manufacture their own fruits or

vegetables at home although, relative to presently used forms of producing fresh produce in mass, the costs of producing a single item would likely be prohibitive. However, as for most technologies, costs tend to become lower over time. Initially, while the prototype is small, the concept would take the form of a table-top printer, using plant suspension cells in liquid medium within cartridges. As the system develops into a robotized format, the printer interface would allow the user to define the desired cell or tissue to be printed, with the desired shape, nutrients, or colours (Fig. 6). Such a concept would benefit tissue engineering science and PTC. In addition, there could be untold benefits of plant bioprinting for the production chain and mass production of rare or valuable plant material. The production in space of plant products rich in nutrients and with reduced volume through plant bioprinting would be suitable where space may be limited, where delivery of renewable resources may be difficult, or impossible, and thus where plant-based resources would be needed to be printed as needed.

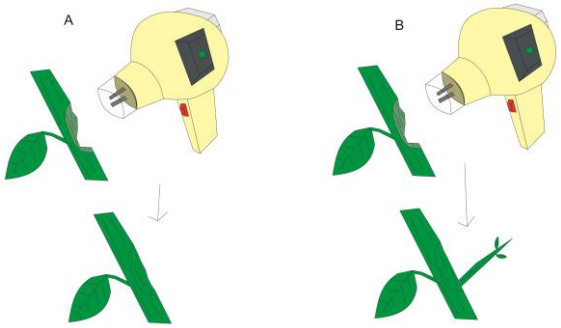


Fig. 5. Handheld plant bioprinter with two applications: (A) healing plant scars and damaged tissue; (B) grafting.

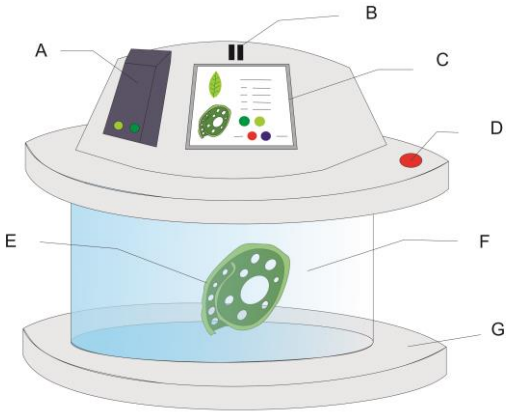


Fig. 6. Future concepts of plant bioprinters for plant-based food. The device consists of a cellular replaceable cartridge (A), USB drive slots (B), a touchscreen display panel (C), a power switch (D), the product to be printed (user interface selection) (E), a print chamber (F), and a device pad that can serve for axillary functions such as medium (scaffold) sterilization, temperature regulation, etc. (G).

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