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*Review*

# Temporary Immersion System for Production of Biomass and Bioactive Compounds from Medicinal Plants

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**Abstract:** The cultivation of medicinal plants and the production of bioactive compounds derived from them are of fundamental importance and interest, not only at the pharmacological level but also in nutraceutical and cosmetic industries and in functional foods, as well as plant protection in agriculture. In order to respond adequately to the increased demands of the global market from a quantitative and qualitative point of view and to guarantee environmental sustainability of the productions, it is necessary to resort to innovation tools, such as tissue culture in vitro technology. Nowadays, it is well known that the cultivation through the Temporary Immersion System (TIS) in a bioreactor has considerable advantages both for the in vitro mass production of the plants and for the production of secondary metabolites. The present review focuses on the application of TIS during the last two decades to produce biomass and bioactive compounds from medicinal plants. Indeed, almost one hundred papers are discussed, and they particularly focus on the effects of the culture system, vessel design and equipment, immersion time and frequency, and substrate composition for 88 medicinal species in TIS bioreactor culture.

**Keywords:** in vitro propagation; plant bioreactors; massive propagation; secondary metabolites; liquid culture; temporary immersion system



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## 1. Micropropagation of Medicinal Plants

Tissues and organs of medicinal plants are a rich source of bioactive compounds, and plant-derived products can be used to improve food quality with healthy ingredients in the form of dietary supplements to contribute to a healthy diet by providing vitamins, antioxidants, and fibers (fortification). Almost 80% of the world's population employs plant-derived components for their health and wellness [1]. The different uses of medicinal plants together with the progress of scientific research allow for new perspectives and promote these species.

The term 'medicinal plant' has been extended in this review to include various uses such as herbal teas, spices, food, dietary supplements, and cosmetics, and it is widely accepted to avoid the narrow focus on modern pharmaceutical applications [2].

Biotechnological tools are important for a higher exploitation of the medicinal plants by adopting techniques such as in vitro regeneration and genetic transformation. In particular, biotechnology by means of tissue culture, cell biology, and molecular biology offers the opportunity to develop plants that are well adapted to changing climates and to meet the market demand [1,3]. In medicinal plants, in order to have standardized formulations the chemical constituents from plants and their parts are required to be uniform both qualitatively and quantitatively. For this reason, clonal mass multiplication is fundamental to yield small quantities of precious active compounds which are required in huge amounts [4].

When possible, medicinal plants should be grown under uniform environmental conditions and have the same genetic origin to ensure the stability of bioactive compounds

and high yield, as in select clones. Frequently, in open field cultivation many medicinal plants show a low rate of seed germination or need special climatic conditions to germinate or take a long time to grow and multiply. Moreover, the productivity of many of these species is low due to the lack of high yielding, biotic stress-tolerant cultivars and the absence of disease-free materials of elite genotypes [5]. Consequently, there is an urgency to preserve and propagate such species for future uses by adopting and improving efficient mass propagation techniques. These goals can be achieved through an in vitro strategy with well-defined protocols and appropriate methods that increase the production of biomass and specific important secondary metabolites. Furthermore, the in vitro propagation technique represents a valid alternative to the collection of resources in the natural environment, preventing the threat of extinction to which many medicinal plants are subjected. Micropropagation of many medicinal plants has been described by various authors since the 1880s [3,6,7]. In the last 20 years in particular, numerous papers have been produced reporting the optimization of micropropagation protocols for medicinal species, mainly the threatened plant species [4,8–10]. The micropropagation of medicinal plants is a tool for producing plants with high-yielding chemotypes for cultivation and industrial purposes, and thus it allows for production of biomass with genetically identical chemotypes and select plants based on the chemical profile in order to standardize a particular chemotype [11]. Finally, micropropagation is useful for reducing consumption pressure on potentially threatened wild populations [11,12].

The propagation of medicinal plants through tissue culture occurs with the typical methods used for other plants. The most common approaches are (i) to isolate organized meristems, like shoot tips or axillary buds, and induce them to grow into complete plants (micropropagation), and (ii) by adventitious shoots from leaf, root, and stem segments or calluses derived from those organs. The last system of propagation involves induction of somatic embryogenesis in cell and callus cultures [3]. Moreover, in vitro tissue culture of medicinal plants is used to produce active compounds for herbal and pharmaceutical industries [13]. The use of medicinal plants in vitro cultures is focused on the production of valuable bioactive compounds [4,14,15] which can be defined as secondary plant metabolites such as pharmaceuticals, flavors, and fragrances. Some metabolites are present in all plants, while others are synthesized in specific species or tissues and sometimes in response to external stimuli.

Large-scale use of plant tissue culture can be an interesting alternative approach to traditional methods of cultivation as it offers a controlled yield and supply of biochemicals independent of plant season availability, resulting in higher production and more consistent quality.

Sometimes the limits to large-scale production are related to the species and others depend on the cost of propagation. Micropropagation by conventional technique is a labor-intensive system of clonal propagation and the automation in bioreactor of micropropagation can be a possible tool for reducing its costs ([14,16–19]. Practically, plant culture bioreactors can be divided into two principal types: bioreactors in which the cultures are continuously submerged in the liquid medium and, more innovatively, those in which the cultures are periodically immersed following specific cycles of time (i.e., temporarily). Hence, this paper focuses on the Temporary Immersion System (TIS) for an overview of its application in medicinal plants during the last 20 years.

## 2. TIS Bioreactors for Micropropagation of Medicinal Plants

The bioreactor is a simple piece of equipment consisting of a culture vessel and a fully automated or semi-automated control system, often with air inflow and outflow systems, designed for intensive culture with the opportunity for monitoring and control of microenvironmental culture conditions. All plant bioreactors use liquid media, either in continuous or in a temporary immersion culture, and this condition offers the best situation for efficient large-scale plant in vitro propagation [20,21]. Indeed, liquid culture systems can provide much more uniform culturing conditions, easy renewal of the media without

changing the container, the possibility of sterilization using microfiltration, and easier cleaning of the container after the culture period.

The production of plant species using a liquid medium in bioreactors is a complementary strategy in order to overcome the limitations present in the in vitro system on conventional semi-solid media. In comparison with culturing on semi-solid media, larger containers can be used, and transfer times can be reduced to avoid intensive manual handling. The bioreactors developed in the past were not suitable for plants micropropagation, they were mainly developed for bacterial culture and did not consider the specific requirements of the plant, such as mechanical damage and foam formation in bubble aerated bioreactors.

The first report on the use of a plant bioreactor in continuous liquid culture for micropropagation was by Takayama and Misawa for Begonia [22]. Sometimes, however, the total permanent immersion of explants into the liquid medium can cause malformations and loss of material due to asphyxia and hyperhydricity [23]. These undesirable physiological conditions are mainly caused by low oxygen rates and water potential of the culture media [23–25].

A TIS bioreactor using the periodical contact between the plants and the liquid medium can be a tool to overcome these difficulties and encourages the development and adaptation of plants to the next stage of acclimatization [26]. This system is based on alternating cycles of temporary immersion of the cultured plant tissue into the liquid medium followed by a dry period. Generally, the immersion period is short (a few minutes), while the dry period is extended (several hours). TIS bioreactors are usually made with transparent glass or plastic vessels, allowing the light from external sources to illuminate the cultivated plant materials. Harris and Mason [27] described for the first time a device that could be reclined to realize temporary immersion in order to combine aeration and liquid medium culture. Various types of TIS bioreactors for plants have been realized, many of them custom-made [28], but currently different TIS models are available on the market consisting of one container (Figure 1) or two containers (Figure 2), and are sometimes equipped for gas exchange by ventilation phases. Detailed descriptions of some plant TIS bioreactors are reported by Georgiev et al. [29], or available on the websites of production companies (e.g., https://setis-systems.be (accessed on 20 November 2021); http://www.plantform.se (accessed on 20 November 2021); http://www.bioreactor-matis.com (accessed on 20 November 2021). The liquid culture in TIS has already been successfully used in the micropropagation of several economically important species, among which are woody plants and ornamental plants [30–33].

**Figure 1.** PlantForm bioreactor TIS model consisting of one container.

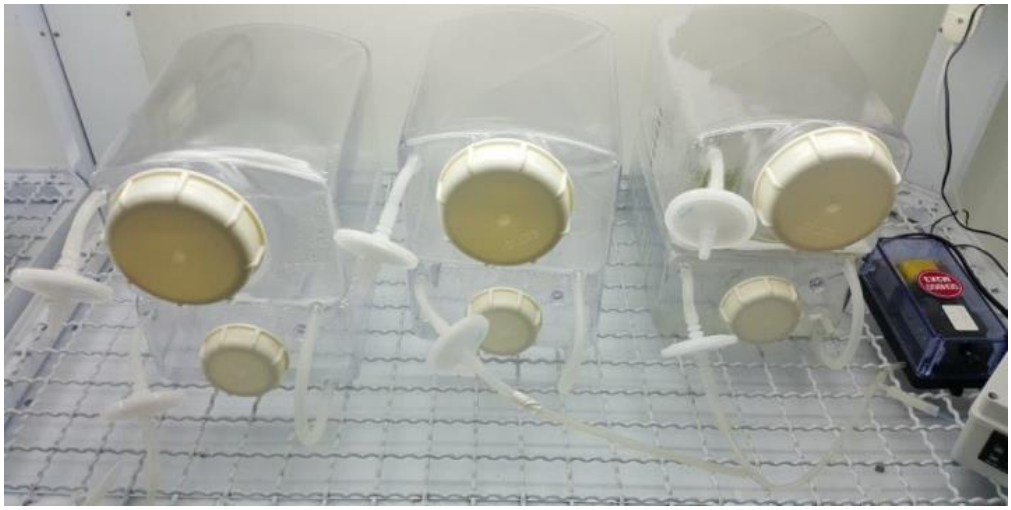

**Figure 2.** SETIS bioreactor TIS model consisting of two containers.

TIS allows for various advantages: (I) a more uniform contact between the culture medium and the plant material in comparison to the conventional culture in semi-solid medium, (II) the reduction of asphyxia and hyperhydricity of cultured tissues, (III) the dilution of toxic compounds (e.g., phenols) released by the plants, producing culture oxidation and browning, (IV) the periodic replacement of the atmosphere within the culture container, which limits gas accumulation (mainly $CO_2$ and ethylene), a typical event which occurs when working with the traditional gas-tight glass jars, (IV) the possibility to extend consistently the subculture time when using large containers, and (V) the possibility to markedly reduce hand labor and cost of production, as a careful positioning of explants in the gelled medium is not necessary and the refill of the fresh liquid medium is done easily.

To develop in vitro plants, the availability of macro- and micronutrients during the culture in bioreactors is essential, influencing both the development and the secondary metabolism production [34]. The optimization of the medium culture (nutrients, carbohydrates, growth regulators) and the control and monitoring of different parameters such as immersion time, temperature, light quality, and aeration, can contribute to producing large amounts of biomass with an increase in the accumulation of secondary metabolites. Moreover, the changing of the immersion program could be used to manipulate the metabolic processes of the in vitro plants, depending on the primary goal of micropropagation or accumulation of specific secondary metabolites [35]. In particular, the precise adjustment of the immersion time and dry periods may significantly reduce the hyperhydricity of the plant tissue, creating conditions for optimal humidity and nutrients supply, a better control of plant morphogenesis, and consequently good biomass [30] and secondary metabolite production. Hyperhydricity is one of the primary factors limiting the liquid culture efficiency, with evident morphological malformation in the cultured plants, such as shorter internodes and brittle, translucent, and wrinkled leaves [36–39].

Overall, the development of medicinal plant production is a key factor for obtaining a commercial source of medicinal biomass for the pharmaceutical industry. In fact, non-stable supply sources can be a bottleneck that limits these beneficial products. For example, fundamental research on anti-cancer pharmaceutical compounds obtained from *Taxus baccata* are restricted to insufficiency in the biomass production and this leads to overharvesting of the natural resources: indeed, the production of 1 kg of taxol needs to collect around 10,000 kg of dry bark of *Taxus* sp. [40]. The advantage of using the TIS in micropropagation comes from the possibility to respond to the high demand of producing plant biomass [41]. TIS is also skilled for secondary metabolite production by differentiated plant tissues and organs [29,30], and it is especially interesting in the medicinal plant sector [21,35,42]. The phytochemicals biosynthesized by the pathways of secondary

metabolism are very important and are required in the pharmaceutical, food, cosmetic, and other industries. The quantity and quality of these bioactive compounds from cultivated or natural plants fluctuate with their composition varies, and for this reason the production by in vitro approaches, in controlled conditions, offers a great prospect. The application of the tissue culture approach for production of secondary metabolites of pharmaceutical importance (particularly alkaloids, steroids, and terpenes) on a commercial scale has been increasing year by year [1]. Despite the growing market of plant metabolites, only a few of them are produced in bioreactors at a commercial scale; the product of the bioreactor unit is the biomass, which then goes through the extraction unit for separation and purification of the target metabolite. The first report on the use of TIS bioreactor for secondary metabolites production was by Schmeda-Hirschmann et al. [43] on *Fabiana imbricata*.

## 3. Use of TIS in Medicinal Plants for the Production of Biomass and Bioactive Compounds from 2000 to Today

The present contribution focuses attention on the application of TIS to medicinal plants over the last 20 years. A bibliometric search showed that during the first decade the average of papers on this topic was low (1.5 papers/year), while in the second decade the number increased four-fold (six papers/year). All the reported papers in this review are divided into two groups based on the final purpose: production of biomass (Table 1) or the accumulation of secondary metabolites (Table 2). Fifty-three species of medicinal plants were propagated with TIS for biomass production, while thirty-six species were considered to obtain specific plant metabolites.

**Table 1.** Biomass production in medicinal plants cultured in TIS with different bioreactors and immersion times. Best results are reported for each species (terminology and values are the same as mentioned by the authors).

| Species | Bioreactor * | Immersion Frequency (Immersion/Dry) | Biomass in TIS (vs. Semi-Solid Culture System) ** | Reference |
|---|---|---|---|---|
| *Anoectochilus formosanus* | Ebb and Flood | 30 min/6 h | Fresh biomass (g/L): ~100 (919.2 in continuous immersion) | [44] |
| *Anthurium andreanum* | RITA | 3 min/3 h | Multiplication rate: 5.17 | [45] |
| | TIB | 2 min/12 h | No. shoots/explant: 50.83<br>FW (g): 0.61<br>DW (g): 0.031 g | [46] |
| | Ebb and Flow | 2 min/4 h | No. shoots/explant: 31.50 (4.50) | [47] |
| *Aquilaria malaccensis* | RITA | 15 min/4 h | Shoots/initial explant: 5.12 (2.14 in continuous immersion)<br>FW (g): 3.12, increases of about 1.8-fold | [48] |
| *Aristotelia chilensis* | Two Vessels | 4 min/6 h | Multiplication rate: 6.38<br>FW (mg) per cluster: 37.42 | [49] |
| *Artemisia judaica* | RITA | 3 min/3 h | Multiplication rate: 65 (35 in continuous immersion) | [50] |
| *Bambusa vulgaris* | Twin Flasks | 2 min/6 h | No. shoots/explant: 13.05 | [51] |
| *Bletilla striata* | BioF-V | 3 min/6 h | *not reported* | [52] |
| *Calathea orbifolia* | Plantima | 2 min/3 h | FW (g): 13.8 (8.2) | [53] |
| *Capparis spinosa* | PlantForm | 2 min/12 h | RGR (mg/g d): 7.89 (0.84)<br>No. shoots/explant: 7.32 (5.24) | [54] |
| *Cattleya walkeriana* | BIT | 3 min/90 min | FW (g): 0.32 (0.24)<br>DW (g): 0.032 (0.008) | [55] |
| *Charybdis numidica* | Two Bottles | 5 min/12 h | Shoot per gram inoculum: 36.6 (194.2) | [56] |
| *Colocasia esculenta* | SETIS | 2 min/4 h | Multiplication rate: 36 (6.10) | [57] |

**Table 1.** *Cont.*

| Species | Bioreactor * | Immersion Frequency (Immersion/Dry) | Biomass in TIS (vs. Semi-Solid Culture System) ** | Reference |
|---|---|---|---|---|
| *Crescentia cujete* | RITA | 3 min/3 h | FW (mg): 950 (200) | [58] |
| *Cymbopogon citratus* | Two Flasks | 6 immersions/24 h | Multiplication coefficient: 12.3 (4.8)<br>FW (g): 66.2 (10.1) | [59] |
| | RITA | 1 min/4 h | *not reported* | [60] |
| *Curcuma longa* | Two Flasks | 4 min/4h | Proliferation rate: 4.2 | [61] |
| *Curcuma zedoaria* | Nalgene® filtration system | 15 min/24 h | No. shoots/explant: 5 (3)<br>FW (g): 4.91 (9.94 in continuous) | [62] |
| *Dianthus caryophyllus* | Two Containers | 2 min/6 h | No. new shoots: 14.33 (5.7) | [63] |
| | RITA | 90 s/8 h | No. shoots/plantlet: 2.0 (1.3) | [64] |
| *Digitalis purpurea* | Two Bottles | 2 min/4 h | FW (g): 104.03<br>DW (g): 5.74 | [65] |
| | Vessels | 2 min/4 h | *not reported* | [66] |
| *Dioscorea fordii* | Plantima | 3 min/4 h | Proliferation rate: 5.0 (2.4)<br>FW (mg): 423.3 (72.2)<br>DW (mg): 39.4 (6.3) | [67] |
| *Echinacea angustifolia* | RITA | *not reported* | Multiplication rate: highest in RITA | [68] |
| *Epipactis flava* | Twin Bottles | 5 min/4 h | No. shoots/explant: 1.5 (1.0) | [69] |
| *Eucalyptus grandis* × *E. urophylla* | TIB | 30 s/3 h | No. shoots/explant: 8 | [70] |
| *Eurycoma longifolia* | RITA | 5 min/4 h | Multiplication rate: 69.6 (26.6) | [71] |
| *Glycyrrhiza glabra* | Twin Flasks | 5 min/4 h | Multiplication rate: 9.47 (6.16) | [72] |
| *Gynura procumbens* | BIT | 15 min/12 h | FW (g): 10.24 | [73] |
| *Helichrysum italicum* | Twin Flasks | 5 min/24 h | Multiplication rate: 16.4 (11.6) | [72] |
| *Hevea brasiliensis* | RITA | 1 min/24 h | RGR: 0.084 (0.085) | [74] |
| *Hippeastrum* × *chmielii* | Twin Flasks | 32 min/24 h | Propagation rate: 6.44 (3.85) | [75] |
| *Hypericum perforatum* | TI Bioreactor | 5 min/3h for 15 days and then 5 min/24 h | No. regenerants/explants: 209 (106, in continuous immersion) | [41] |
| *Hypoxis argentea* | PlantForm | 6 min/12 h | No. shoots/explant: 12 | [76] |
| *Jacaranda decurrens* | RITA | 15 min/4 h | Multiplication rate: 9.61 | [77] |
| *Jeffersonia dubia* | Plantima | 30 s/30 min | No. shoots/explant: 13.6 (7.3) | [78] |
| *Lessertia (Sutherlandia) frutescens* | BTBB | 30 min/4 h | Shoot multiplication: 12.9 (7.8)<br>FW (mg): 3109 (104.2)<br>DW (mg): 351.8 (10.3) | [79] |
| *Leucojum aestivum* | RITA | 5 min/2 h | No. bulbs (%): 74.6 (71.7) | [80] |
| *Morinda royoc* | TIS Bioreactor | 2 min/4 h | Multiplication coefficient: 6.0 | [81] |
| *Myrtus communis* | PlantForm | 8 min/16 h | RGR: 6.2 (5.3) | [82] |
| | PlantForm | 15 min/8 h | Micropropagation rate: 11.40 (6.25)<br>FW (g): 1.00 (0.17)<br>DW (g): 0.26 (0.02) | [83] |
| *Olea europaea* | PlantForm | 8 min/16h | No. shoots/explant: 0.77 (0.36) | [84] |
| *Picea abies* | PlantForm | 20 min/4 h | Embryo production per g pro-embryogenic mass: 696 (174) | [85] |
| *Picrorhiza kurroa* | Twin Flasks | 5 min/8 h | Multiplication rate: 8.20 (4.68) | [72] |

**Table 1.** *Cont.*

| Species | Bioreactor * | Immersion Frequency (Immersion/Dry) | Biomass in TIS (vs. Semi-Solid Culture System) ** | Reference |
|---|---|---|---|---|
| *Pinellia ternata* | *not reported* | *not reported* | No. tubers/plantlet: 24.73 (14.75) | [86] |
| *Piper aduncum* | RITA | 3 min/6 h | Germination of somatic embryos (%): 100 (100) | [87] |
| *Pistacia lentiscus* | RITA | 3 h/24 h | No. shoot/explant: 6.18 (5.8) | [88] |
| *Psidium guajava* | RITA | 2 min/6 h | Multiplication coeff.: 4.90 (4.36)<br>No. shoots: 2.17 (1.78) | [89] |
| *Quercus robur* | PlantForm | 8 min/16 h | RGR: 6 (4)<br>No. shoots/explant: 3 (2.5) | [90] |
| *Rhodiola rosea* | RITA | 15 min/4 h | No. shoots/explant: 16 | [91] |
| *Saponaria officinalis* | Twin Flasks | 5 min/8 h | Multiplication rate: 4.36 (2.39) | [72] |
| *Siraitia grosvenorii* | Plantima | 4 min/4 h | Proliferation rate: 8.75 (3.72)<br>FW (mg): 501.25 (168.89)<br>DW (mg): 60.83 (21.94) | [92] |
| *Stevia rebaudiana* | BIT | 10 min/12 h | FW (mg): 774.39<br>DW (mg): 83.41 | [93] |
| | RITA<br>PlantForm | 3 min/3 h<br>3 min/8 h | No. shoots/explants: 14 | [94] |
| | RITA | 2 min/4 h | No. shoots/explant: 11.8 (4.6) | [95] |
| | BIT | 2 min/12 h | Multiplication rate: 8 shoots/plant | [96] |
| | RITA | 10 s/1 h | No. shoots/explant: 8.47 (2.00) | [97] |
| *Swertia chirayita* | Twin Flasks | *not reported* | No. shoots/explants: 28 | [98] |
| *Tropaeolum tuberosum* | RITA | 2 min/3 h | Microtubers quantity: 56<br>Weight of microtubers (g): 0.2<br>Size of microtuber (cm): 1.9 | [99] |
| *Tussilago farfara* | Two Flasks | 5 min/8 h | Multiplication rate: 5.65 (4.15) | [72] |
| *Vaccinium corymbosum* | Two Vessels | 3 min/6 or 8 h | Multiplication rate: 26.2 (12.7) | [100] |
| *Vanilla planifolia* | BIT | 2 min/4 h | No. shoots/explants: 18.06<br>FW (g): 6.02<br>DW (g): 0.41 | [95] |
| | TIB | 2 min/8 h | No. shoots/plantlet: 9.15 (1.3) | [46] |
| | RITA | 2 min/4 h | Multiplication rate: 14.27 (5.80) | [101] |
| | RITA | 2 min/6 h | No. shoots/explant: 14.89<br>FW (g): 5829.00<br>DW (g): 447.20 | [102] |
| | SETIS | 2 min/4 h | No. shoots/explant: 11.41 (3.76)<br>FW (g): 6.65 (1.71)<br>DW (g): 0.42 (0.15) | [103] |
| *Zingiber zerumbet* | Nalgene® filtration system | 15 min/24 h | No. shoots/explant: 4 (3)<br>FW (g): 2.31 (9.94 in continuous immersion) | [62] |

* Different names of Twin Flasks (two vessels, two bottles, two containers, twin bottles) refer essentially to analogous TIS systems. ** Values between parentheses refer to the semi-solid culture, if not differently specified. FW: fresh weight, DW: dry weight, BioF-V: TIS bioreactor from Biofunction Co. Ltd. (Nanjing, China), RGR: Relative Growth Rate, Plantima: TIS bioreactor from A-Tech Bioscientific Co., Ltd. (Taipei, Taiwan), BTBB: balloon-type bubble bioreactors.

The effects of the culture system, vessel design, equipment, immersion time and frequency, and substrate composition were investigated. It should be noted that in the examined papers the values were not always consistent with each other; for example, the

biomass can be expressed as the number of shoots per explant, fresh or dry weight, or multiplication rate. Therefore, the values of biomass production in Table 1 and of bioactive compounds in Table 2 are compared with the values obtained in semi-solid medium; if not available, the culture system used for the comparison is reported.

### *3.1. Culture System*

In the literature, the TIS guarantees better nutrients and Plant Growth Regulators (PGRs) uptake, as well as higher daily air exchange in comparison to semi-solid culture media [104]. Therefore, several studies evaluated the effectiveness of TIS and other culture systems on achieving the maximal biomass production (Table 1) and optimizing the accumulation of bioactive compounds (Table 2).

#### 3.1.1. Culture System and Biomass Production

Some species have adapted very well to propagation in TIS compared to culture on semi-solid medium, such as *Dianthus caryophyllus*, which recorded an average of 3.0 shoots per plantlet instead of 1.3 and fresh weight (FW) at least 3 times greater (with frequency immersion of 90 s/8 h) than conventional culture in semi-solid medium [64].

*Pistacia lentiscus*, *Stevia rebaudiana*, and *Vanilla plantifolia* showed a significantly improved multiplication rate when grown in TIS compared to conventional semi-solid culture system [88,95,101]. In particular, shoots of *Vanilla plantifolia* produced the maximum multiplication rate (14.27 shoots per explant) when using TIS, with an immersion frequency of 2 min every 4 h, i.e., 2.5 times higher than the multiplication rate on semi-solid medium (5.80 shoots for explant). Interestingly, *Crescentia cujete* inoculated in a TIS bioreactor had a higher significant fresh and dry weight than those in semi-solid and flask-liquid culture [58].

It was possible to promote the growth and quality of *Siraitia grosvenorii* and *Dioscorea fordii* plantlets by TIS, which reported a greater proliferation rate, FW, and dry weight (DW), while in semi-solid and liquid the medium decreased [67,92]. *Morinda royoc* [81] and *Psidium guajava* [89] explants growth in TIS recorded the highest values of multiplication coefficient and number of shoots with maximal biomass compared with semi-solid culture.

The efficiency of TIS was noted when a considerable increase in multiplication rate was observed in *Echinacea angustifolia* [68] and *Jacaranda decurrens* [77]. For the micropropagation of blueberry plants, a promised approach mediated by TIS doubled the multiplication rate values compared to agar-base medium [100]. Similarly, TIS in *Vanilla planifolia* maximized the multiplication rate (14.27 shoots vs. 5.80 in semi-solid) [101], the FW (6.65 g vs. 1.71 in semi-solid), the DW (0.42 g vs. 0.15 in semi-solid) [103], as well as in *Anthurium andreanum* the shoot number (31.50) per plant [47], with respect to the partial immersion system (7.25), where about 5 mm of the shoot base was submerged in liquid medium and semi-solid medium (4.50).

The comparison of three different culture systems showed the prevalence of TIS over conventional semi-solid and liquid continuous immersion to induce the highest shoot number (1.5) of *Epipactis flava* [69] and to obtain the largest FW (3109.0 g) and DW (351.8 g) of *Lessertia frutescens* [79]. In addition, the application of TIS treatments on *Hippeastrum* × *chmielii* yielded greater propagation rates (6.44) than semi-solid medium (3.85) [75]. Aka Kaçar et al. [83] referred to TIS as a valid alternative to traditional in vitro micropropagation of myrtle, resulting in plantlets successfully growing better than those in semi-solid medium in terms of FW and DW.

*Olea europaea*, an important species for the Mediterranean countries, was tested under TIS conditions. Indeed, an efficient proliferation of shoots was reported per explant (0.77), whereas it was reduced to 0.36 shoots in the semi-solid culture [84]. To scale up the production of *Picea abies*, a 2.5-fold increase in the pro-embryogenic mass was achieved in the TIS bioreactor compared to semi-solid medium [85]. The micropropagation of *Pinellia ternata* was improved using the temporary immersion bioreactor, as it showed a high potential to induce the largest number of tubers per plantlet compared to solid and

liquid culture [86]. On the other hand, no difference between the semi-solid medium and TIS was noted for *Quercus robur* production with respect to the shoot number per explant. Yoon et al. [44] revealed that biomass production from *Anoectochilus formosanus* was less efficient when culturing was performed under temporary immersion. In a recent study on in vitro multiplication of *Colocasia esculenta*, applying different culture systems demonstrated that the highest regeneration rate was with TIS [57].

#### 3.1.2. Culture System and Bioactive Compounds Content

To meet the increasing market demand for rapid propagation of plant material and then a stable supply of secondary metabolites, many studies based on in vitro culture methods have been performed to improve the production of specific plant-derived chemicals.

Plants from *Arnica montana* showed higher sesquiterpene lactones content (15.34 mg/g DW) in TIS than those from the semi-solid medium (7.62 mg/g DW) and static liquid medium (13.81 mg/g DW) [105]. The level of camptothecin (2.5 mg/g DW) of *Camptotheca acuminata* grown in TIS was comparable to the semi-solid culture [106]. In contrast, plumbagin, as the main bioactive compound from *Drosera communis*, was reduced in its yield by TIS compared to the semi-solid system and continuous immersion system [69]. Shoots of *Lycium barbarum* propagated in TIS were characterized by high concentration of total phenolic (23.6 mg gallic acid equivalent-GAE/g DW) and low flavonoid content (1.9 mg rutin equivalent-RE/g DW) compared to semi-solid culture [107]. The effect of TIS on the growth, development, quality, and biochemical characteristics of different rose species was investigated by Malik et al. [108], showing that *Rosa tomentosa* and *Rosa rubiginosa* plants propagated in TIS were characterized by a high content of phenolic compounds, soluble sugars, and multiplication rate, and were better than those of plants developed on semi-solid culture.

Interestingly, the contents of major bioactive compounds in *Hypericum perforatum* (Hypericin, 0.18 mg/g DW), *Cymbopogon citratus* (α-citral, 0.35 mg/g DW and β-citral, 0.54 mg/g DW), and *Fabiana imbricata* (oleanolic acid, 0.01 mg/g DW) were considerable in plant material grown in TIS compared to cell suspension and callus cultures [109]. Since flavonoids are the principal active constituents of *Scutellaria baicalensis*, six culture systems were compared to optimize the phytochemical content in plant tissues. Indeed, TIS was effective in providing significantly higher baicalein and baicalin concentration than the other systems [110]. Vives et al. [111] showed a considerable increase in the formation of steviol glycosides (0.12 g) as natural sweeteners from *Stevia rebaudiana* shoots in TIS compared to liquid (0.02 g) and semi-solid (0.03 g) culture medium.

TIS promoted the contents of carotenoid (0.40 µg/mL) in wild *Vaccinium vitis-idaea* ssp. *Minus* more than shoots micro-propagated in stationary bioreactor [112]. Adventitious roots of *Catharanthus roseus* grown in the temporary immersion bioreactor offered high ajmalicine content [113]. In vitro cultures of *Harpagophytum procumbens*, the plants grown using TIS accumulated total iridoids, particularly harpagoside and its concentration in the leaves, were significantly greater than the control treatment [114].

It was possible to scale up the production of *Rhododendron tomentosum* using various liquid systems to improve the accumulation of bioactive volatile fractions. The highest essential oil content and its terpenoid compounds, mainly *p*-cymene, alloaromadendrene, shyobunone, and ledene oxide II, were obtained by means of TIS [115]. Mišić et al. [116] hypothesized that higher biomass and metabolites in hair roots of *Centaurium maritimum* were obtained in TIS bioreactors as result of high $O_2$ transfer rates, reduced shear and hydrodynamic forces, better nutrient availability, and avoidance of hyperhydricity phenomena.

**Table 2.** Bioactive compounds production in medicinal plants cultured in TIS with different bioreactors and immersion times. Best results are reported for each species (terminology and values are the same as mentioned by the authors).

| Species | Bioreactor * | Immersion Frequency (Immersion/Dry) | Bioactive Compound in TIS (vs. Semi Solid Culture System) ** | Reference |
|---|---|---|---|---|
| *Arnica montana* | RITA | 5 min/6 h | Sesquiterpene lactones (mg/g DW): 15.34 (7.62) | [105] |
| *Aristotelia chilensis* | Two Vessels | 4 min/6 h | Total phenols (g GAE/100 g DW): 2.97 (3.04 in wild plants) | [49] |
| *Beta vulgaris* | RITA | 15 min/1 h | Betalains (mg/g DW): 18.8<br>Betacyanins (mg/g DW): 9.6<br>Betaxanthins (mg/g DW): 9.2 | [117] |
| *Camptotheca acuminata* | DVS, RITA | 1 min/3 h | Camptothecin (mg/g DW): 2.5 (2.2) | [106] |
| *Castilleja tenuiflora* | RITA | 30 min/24 h | Total phenolics (mg GAE/g DW): 30.58<br>Total flavonoid (µg CE/g DW): 45.83 | [118] |
| | RITA | 5 min/24 h | Total phenolics (mg GAE/g DW): 41.3<br>Verbascoside (mg/g DW): 113.9<br>Isoverbascoside (mg/g DW): 36.4 | [119] |
| *Catharanthus roseus* | Bottles | 5 min/3 h | Ajmalicine (µg/g DW): 950 | [113] |
| *Centaurium maritimum* | RITA | 15 min/45 min | Total secoiridoid glycosides: 8 times higher vs. liquid culture | [116] |
| *Centella asiatica* | PlantForm | 5 min/90 min | Asiaticoside (mg/g DW): 7.91 (4.45 in agitated flasks)<br>Madecassosid (mg/g DW): 3.81 (2.40 in agitated flasks) | [120] |
| *Chlorophytum borivilianum* | RITA | 15 min/1 h | Total saponin (mg diosgenin equiv./g DW): 21 (16 in mother plants) | [121] |
| *Cymbopogon citratus* | Twin Vessels | 5 min/4 h | α-citral (mg/g DW): 0.35 (0.27)<br>β-citral (mg/g DW): 0.54 (0.46) | [109] |
| *Digitalis lanata* | Two Vessels | 2 min/4 h | Lanatoside C (µg/g DW): 316 | [122] |
| *Digitalis purpurea* | Two Vessels | 2 min/4 h | Digitoxin (µg): 167.6<br>Digoxin (µg): 119.9 | [123] |
| *Dracocephalum forrestii* | RITA | 10 min/80 min | Chlorogenic acid (mg/g DW): 0.99 (0.88 in NSB)<br>Acacetin rhamnosyl-trihexoside (mg/g DW): 1.01 (0.91 in NSB)<br>Rosmarinic acid (mg/g DW): 11.91 (18.35 in NSB)<br>Acacetin acetylrhamnosyl-trihexoside (mg/g DW): 2.45 (2.25 in NSB)<br>Apigenin p-coumaroylrhamnoside II (mg/g DW): 2.28 (1.76 in NSB) | [124] |
| *Drosera communis* | Twin Bottles | 5 min/4 h | Plumbagin content (µg/g DW): 2.44 (4.22) | [69] |
| *Fabiana imbricata* | Twin Vessels | 5 min/4 h | Oleanolic acid (mg/g DW): 0.01 (0.01) | [109] |
| | Bottles | 10 min/4 h | Oleanolic acid (% DW): up to 0.14% (3.47 in wild plant)<br>Rutin (% DW): 0.20 (3.35 in wild plant) | [43] |
| *Gynura procumbens* | BIT | 15 min/12 h | Flavonoids content mg (CE/g DW): 32.0 | [125] |

**Table 2.** *Cont.*

| Species | Bioreactor * | Immersion Frequency (Immersion/Dry) | Bioactive Compound in TIS (vs. Semi Solid Culture System) ** | Reference |
|---|---|---|---|---|
| *Harpagophytum procumbens* | Two Erlenmeyer Flasks | 60 min/24 h | Harpagoside (mg/gDW): Leaves: 6.0 (3.5 in glasshouse grown plants) Stem: 3.8 (5 in glasshouse grown plants) Root: 8 (2 in glasshouse grown plants) | [114] |
| *Hypericum perforatum* | Twin Vessels | 5 min/4 h | Hypericin (mg/g DW): 0.18 (0.15) | [109] |
| *Lavandula officinalis* | Twin Vessels | 5 min/4 h | Rosmarinic acid (mg/g DW): 5.7 (55.3) | [109] |
| *Leucojum aestivum* | Bioreactor Vessels | 5 min/2 h | Galanthamine (mg/l): 4.64 | [126] |
| | RITA | 15 min/8 h | Galanthamine (µg/RITA): 265 Lycorine (µg/RITA): 1.699 Norgalanthamine (µg/RITA): 225 | [127] |
| *Lycium barbarum* | PlantForm | 6 min/24 h | Total phenol (mg GAE/g DW): 23.6 (19.4) Total flavonoid (mg RE/g DW): 1.9 (18.5) | [107] |
| *Nasturtium officinale* | RITA | *not reported* | Total polyphenols (mmol TE/100 g DW): 3.74 (2.70 in mother plants) Total flavonoids (mmol RE/100 g DW): 1.64 (1.89 in mother plants) Total glucosionolates (mg/100 g DW): 261.97 (799.47 in mother plants) | [128] |
| *Panax ginseng* | RITA | 5 min/1 h | Saponin (mg/g DW): 15.94 | [129] |
| | RITA | 5 min/1 h | Saponin (mg/g DW): 28.51 | [130] |
| *Pancratium maritimum* | RITA | 15 min/12 h | Alkaloid (µg/g DW): 3.469 Haemanthamine (µg/g): 900.1 Lycorine (µg/g): 799.9 | [131] |
| *Rhododendron tomentosum* | RITA | 5 min/85 min | p-cymene (%): 6.9 (0.9) Alloaromadendrene (%): 5.5 (8.1) Shyobunone (%): 8.2 (15.8) Ledene oxide (II) (%): 13.0 (14.7) | [115] |
| *Rosa canina* | RITA | 15 min/12 h 15 min/6 h 15 min/8 h | Total phenolics (mg/g FW): 15.8 (11.4) Soluble sugars (mg/g FW): 9.2 (8.0) Carotenoids (mg/g FW): 0.66 (0.99) | [132] |
| *Rosa rubiginosa* | RITA | 15 min/8 h | Total phenolics (mg/g FW): 8.03 (9.57) Carotenoids (mg/g FW): 0.10 (0.17) Soluble sugars (mg/g FW): 9.41 (7.99) | [108] |
| *Rosa rugosa* | BTBB | *not reported* | Total phenolics (mg/g DW): 10 (22) Total flavonoids (mg/g DW): 3 (3.5) | [133] |
| *Rosa tomentosa* | RITA | 15 min/8 h | Total phenolics (mg/g FW): 9.91 (9.66) Carotenoids (mg/g FW): 0.11 (0.09) Soluble sugars (mg/g FW): 7.34 (9.39) | [108] |

**Table 2.** *Cont.*

| Species | Bioreactor * | Immersion Frequency (Immersion/Dry) | Bioactive Compound in TIS (vs. Semi Solid Culture System) ** | Reference |
|---|---|---|---|---|
| *Salvia viridis* | PlantForm | 10 min/80 min | Total phenolic acid (mg/g DW): 18.3<br>Total phenylethanoid (mg/g DW): 11.4<br>Total phenol (mg/g DW): 29.7 | [134] |
| *Schisandra chinensis* | PlantForm | 5 min/90 min | Lignans (mg/100 g DW): 546.98 (185.77 in agitated cultures)<br>Schisandrin (mg/100 g DW): 118.59<br>Deoxyschisandrin (mg/100 g DW): 77.66<br>Gomisin A (mg/100 g DW): 67.86 | [135] |
| | PlantForm, RITA | 5 min/90 min | Total phenolic acids (mg/100 g DW): 34.56 in PlantForm (46.68 in continuous immersion)<br>Total flavonoids (mg/100 g DW): 21.27 in RITA (29.02 in continuous immersion) | [136] |
| *Scutellaria baicalensis* | RITA | 5 min/3 h | Baicalein (µg/mg DW): 2.2 (1.1)<br>Baicalin (µg/mg DW): 3.0 (1.0)<br>Wogonin (µg/mg DW): 0.10 (0.25) | [110] |
| *Stevia rebaudiana* | BIT | 3 min/6 h | Total steviol glycosides/container (g): 0.1698 (0.0239) | [111] |
| *Thapsia garganica* | RITA | 3 min/6 h | Thapsigargin (mg/g DW): 2.15<br>Nortrilobolide (mg/g DW): 17.42 | [137] |
| *Vaccinium vitis-idaea* ssp. *minus* | RITA | *not reported* | Carotenoids (µg/mL): 0.40 (0.30 in stationary bioreactor) | [112] |
| *Zeltnera beyrichii* | RITA | 15 min/6 h | Secoiridoid glycoside (mmol per 100 g DW): 7.5 (30) | [138] |

* Different names of Twin Flasks (bottles, two vessels, twin bottles, twin vessels) refer essentially to analogous TIS systems. ** Values between parentheses refer to the semi-solid culture, if not differently specified. DW: dry weight, DVS: Dual-Vessel System, NSB: Nutrient Sprinkle Bioreactor; FW: fresh weight, BTBB: Balloon-Type Air-Lift Bubble Bioreactor.

### *3.2. Type of TIS Bioreactors*

Over last 20 years, the RITA® system has been the most used bioreactor (39.8% of the reported papers) to produce biomass (31.3%) and metabolites (53%) (Figure 3); it has also been the first bioreactor to be designed and sold on the market by a commercial company (Vitropic, France, https://www.cirad.fr accessed on 20 November 2021). Different plant species have responded successfully to the RITA system, such as vanilla [101], pistachio [32], and cork oak [139]. Following the Twin-Flask system is one of the earliest developed TIS [140], and it was used in 24% of the papers. This model has a simple operation and often has been, or is still, custom-made in the laboratory and is called by various names (e.g., BIT®, TIB, two vessels, two bottles, etc.).

The RITA and the Twin-Flask bioreactors are widely used for metabolite production (53% and 24%, respectively; Figure 3). BIT or TIB (Temporary Immersion Bioreactor; [140]), a registered trademark bioreactor working on the principle of Twin-Flask containers, was present in 12% of applications for biomass and 5% for bioactive compounds production. PlantForm™ bioreactor (http://www.plantform.se/ (accessed on 20 November 2021) was proposed more recently and, for this reason, only 13 papers reported its application. Plantima, Ebb, and Flow devices [29] are used in medicinal plants only for mass propagation. Other types of bioreactors are applied in biomass (7.5%) and metabolites yield (7%), among which is the SETIS™ (https://setis-systems.be (accessed on 20 November 2021), a bioreactor of the last generation that has proven to be very promising in various species [141–143].

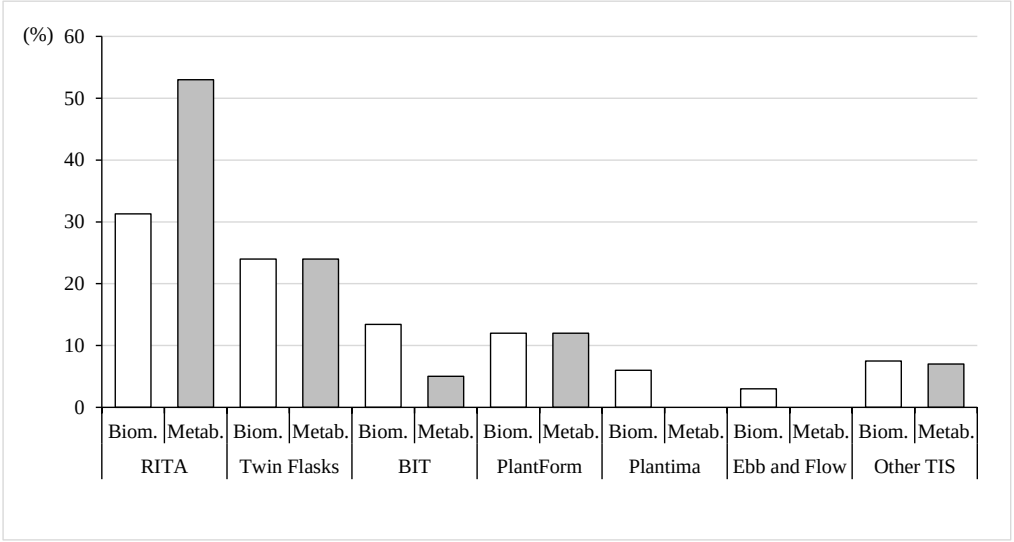


**Figure 3.** TIS bioreactors application percentage in the reported papers for biomass (61 papers) and bioactive compounds production (36 papers).

The specific design of a given TIS has a direct effect on the in vitro development of plants [29]. This is mainly because each system provides different physical culture conditions which, in turn, have a direct impact on the in vitro environment, light availability, space, water, and nutrients [29,135]. RITA, Twin Flasks, and PlantForm differ mainly in the volume of the containers and type of ventilation, being passive in the first two and forced in the last. Moreover, TIS design and materials influence the transmission of light to plant [29] and light irradiance directly affects the synthesis of photosynthetic pigments and the photosynthetic rate of plants cultivated in vitro [144]. Although the different effectiveness is evident in the literature, only a few papers investigated the effect of various types of TIS on the propagation and the production of metabolites in the same species.

#### 3.2.1. Types of TIS Bioreactors and Biomass Production

Different types of TIS were compared in *Anthurium andreanum* during the in vitro propagation. The largest number of shoots per explant was 50.83 and 43.16 with TIB and Ebb and Flow, respectively, compared to 30.66 with RITA; moreover, in TIB the shoots showed a greater photosynthetic efficiency [46] (Table 1). Additionally, other studies on the micropropagation of anthurium reported 31.50 shoots per explant with the Ebb and Flow bioreactor [47] and a multiplication rate of 5.17 with the RITA system [45].

The liquid culture in PlantForm or RITA bioreactors was found to be efficient for *Stevia rebaudiana* propagation, with the number of shoots per explant equal to 14, as well as for its rooting phase [94]. The authors noted that, although the PlantForm has a larger space to allow for material enlargement rather than RITA, it is necessary to carefully set the flooding conditions with the PlantForm system to obtain good quality plants suitable for the acclimatization phase in greenhouse; flooding every 8 h guaranteed a better behavior and quality without hyperhydricity. A smaller number of explant per shoots (11.8 and 8.47) was obtained by Ramírez-Mosqueda and Iglesias-Andreu [95] and Bayraktar [97], respectively, always using the RITA for the micropropagation of stevia. Furthermore, three TIS bioreactors, SETIS, RITA and BIT, were compared for the propagation of two stevia cultivars and their performance in the field was evaluated (Rosales et al., 2018). Each system produced vigorous plants with low levels of hyperhydricity, great average numbers of leaves and shoots, and a higher rate of multiplication than those explants cultured in semi-solid medium. In particular, the BIT container showed the best results in terms of plant elongation and shoots per explant. The efficacy of BIT documented in some species, including vanilla [95], is due to the easy availability of components in the laboratories and

easy assembling [29,145]. Ramirez-Mosqueda and Iglesias-Andreu [95] compared three different bioreactors during the multiplication and the rooting of *Vanilla planifolia*: BIT, Gravity Immersion Bioreactors (BIG), and RITA. A higher number of shoots per explant (18.06) was obtained in the multiplication phase in BIT systems, followed by RITA (12.77) and BIG (6.83). In addition, the fresh and dry weights of shoots and the number of longer roots were higher in the BIT compared with the other systems. The efficiency of the RITA system in vanilla propagation was also evaluated by Ramos-Castellà et al. [101] and Spinoso-Castillo et al. [102], which obtained 14.27 as the multiplication rate and 14.89 shoots per explant, optimizing two types of immersion.

#### 3.2.2. Type of TIS Bioreactors and Bioactive Compounds Content

The selection of bioreactor type and optimization of process parameters are decisive for maximizing secondary metabolite production [19,21,110,146]. Many kinds of bioactive compounds have been explored and produced in different types of bioreactors (Table 2). Total phenolics, total flavonoids, and total carotenoids from different medicinal plants were the main groups of metabolites studied in TIS. The role of plant-derived phenolic substances in various areas such as medicine, cosmetology, and food industry is precious, and compounds like flavonoids and phenolic acids with scientific evidence are used for many important biological activities as antioxidants [147,148], anti-inflammatories [149], or anticancer properties [150]. The RITA system is the bioreactor most used in the production of phenols and flavonoids, such as in *Castilleja tenuiflora*, *Nasturtium officinale*, *Schisandra chinensis*, and *Zeltnera beyrichii.* In particular, a higher total polyphenol content (3.74 mmol trolox equivalent-TE/100 g DW) was obtained from *Nasturtium officinale* microshoots cultured in RITA system over 10 days, which was 1.4-fold higher in comparison to the herb extracts from the mother plants, while the total flavonoid content was 1.64 mmol RE/100 g DW after 10 days of growth in RITA bioreactor respect to 1.89 mmol RE/100 g DW in the herb extract from mother plants [128]. Some papers report the production of phenolics from *Rosa* spp. High contents were obtained for *R. canina* (15.8 mg/g FW), *R. rubiginosa* (8.03 mg/g FW), and *R. tomentosa* (9.91 mg/g FW) by the application of the RITA system [108,132]. Using a Balloon-Type Air-Lift Bubble Bioreactor (BTBB) adapted for temporary immersion culture in *R. rugosa* led to less total phenolics and total flavonoids compared to a continuous immersion bioreactor [133].

The influence of bioreactor type on the accumulation of phenolic acids and flavonoids in microshoot cultures of *Schisandra chinensis* was proven and optimized when comparing five different bioreactors: three with continuous immersion and two with temporary immersion [136]. The tested TIS bioreactors, RITA and PlantForm, were always better than the continuous immersion system in relation to biomass productivity, while the continuous immersion systems showed the best results in terms of phenolic acids and flavonoids contents. Taking into account only TIS systems, small differences were recorded in the accumulation of two groups of phenolic compounds: indeed, the production of phenolic acids was 34.56 mg/100 g DW in PlantForm and 32.65 mg/100 g DW in RITA, and the one with flavonoids was 21.27 mg/100 g DW in RITA and 20.48 mg/100 g DW in PlantForm [136]. In the same species, PlantForm and RITA systems were compared for the production of lignans [135]. Best results were obtained by the PlantForm bioreactor (546.98 mg/100 g DW), in terms of the main lignan constituents: schisandrin, deoxyschisandrin, and gomisin A.

Total phenols and flavonoids were recorded in vitro culture of *Lycium barbarum* cultivated in PlantForm [107]. Here, the TIS culture resulted in an increase of the total phenolic content and a lower value of the total flavonoid content, with respect to the control with a semi-solid culture system. Superior results were obtained in *Salvia viridis* using PlantForm as TIS bioreactor in comparison to in vivo plants. Indeed, after three culture weeks, the total phenolic acids level was almost 10 times higher than in four-month-old plants growing in the soil [134]. *Zeltnera beyrichii* plants grown in a RITA bioreactor contained moderate levels of total phenolics [138].

The PlantForm bioreactor turned out to be the best system in terms of phenolic acids, flavonoids, and centellosides productivity in *Centella asiatica* [120] compared to the RITA bioreactor and agitated flasks in the continuous system. The production of total phenolic compounds, mainly chlorogenic acid and rosmarinic acid, was recorded in *Dracocephalum forrestii* shoots, a tibetan medicinal plant, cultured in RITA bioreactor. However, the conditions were different for flavonoid glycosides, where PlantForm was more effective for the production of the apigenin p-coumaroyl-rhamnoside II and acacetin acetylrhamnosyl-trihexoside [124]. Rosmarinic acid, a bioactive compound with the most prominent antibacterial, anti-inflammatory, and antiviral activities [151], was also produced from *Lavandula officinalis* using the Twin-Flask system, although the amount was higher in semi-solid culture [109].

Another important group of bioactive substances, the carotenoids, were produced in TIS. Carotenoids, as potent antioxidants, are recognized as playing an important role in the prevention of human diseases and the maintenance of good health (Rao and Rao, 2007). In *Rosa canina* [132], *R. rubiginosa*, and *R. tomentosa* [108], the carotenoid content was 0.66, 0.10, and 0.11 mg/g FW, respectively, when this species was propagated in the RITA system, while in the same type of bioreactor *Vaccinium vitis-idea* produced 0.40 µg/mL of carotenoids [112].

In the RITA bioreactor, an eight-times higher total secoiridoid glycosides production rate was achieved in *Centaurium maritimum*, a plant which contains a considerable amount of bioactive compounds [116]; in addition, a high saponin content was found in adventitious roots *Panax ginseng* cultivated in RITA [130]. It is also interesting to note that the shoot culture in two vessels of *Digitalis purpurea* promoted both a high biomass production and a high of cardiotonic glycoside [123]. Recently, in maqui (*Aristotelia chilensis*) the contents of metabolites in TIBs did not differ from those of wild plants [49]. The authors suggest some observations for the scaling up to SETIS bioreactors as a system to improve the production efficiency of phenylpropanoids.

### *3.3. Immersion Frequency*

In every system of TIS liquid culture, the selected cycle of immersion is the most critical parameter for the system efficiency. Tables 1 and 2 show a considerable variation of immersion times, ranging from hours to a few seconds due to the large variety of species, micropropagation processes, and TIS used.

#### 3.3.1. Immersion Frequency and Biomass Production

An immersion frequency of 30 s every 30 min or every 3 h produced the highest number of shoots per explant in *Jeffersonia dubia* [78] and in *Eucalyptus grandis* × *E. urophylla* ([70] (Table 1). The effect of immersion interval (15 min immersion every 4 h) was more critical in *Aquilaria malaccensis* shoot cultivation when an increase in the number of shoot regeneration (5.12) and biomass (3.12 g) were obtained compared to 5 min/4 h [48].

In order to increase the regeneration rate of *Charybdis numidica*, the immersion frequency was doubled from 5 min/24 h to 5 min/12 h. However, it turned out that the shoots became very sensitive to the prolonged contact with the nutrient medium, resulting in completely hyperhydrated shoots [56]. The effect of TIS at two, four, and six immersions per day on the biomass production of *Cymbopogon citratus* was studied. The maximum values of FW (66.2 g) were obtained in the treatments of four and six immersions per day, whereas the values of DW (6.4 g) were greater in the frequency of four immersions per day [59]. Significant and distinctive differences were recorded by Ahmadian et al. [63] in vitro *Dianthus caryophyllus* propagation using TIS. Although increasing the immersion times induced the greatest number of new shoots, the highest rate of hyperhydricity was observed. Since hyperhydricity is a remarkable sign of inefficiency of TIB, the most desirable treatment was four immersions of 2 min/day with 14.33 new shoots. In a later study on the same species, incidence of hyperhydricity was greater (62%) in plantlets cultured with the 2-h dry phase and reduced to 16% when the dry phase increased to 8 h with

60 s [64]. However, the best results suggest that carnation in TIS with 90 s immersion every 8 h had superior growth traits, normal shapes, a higher number of stomata, and total absence of hyperhydricity.

The influence of immersion frequency and duration on in vitro propagation of medicinal and aromatic plants was investigated by Wawrosch [72]. An immersion setting of 5 min/4 h was proven to enhance the multiplication rate of *Glycyrrhiza glabra* (9.47), while 5 min/8 h improved the multiplication of *Saponaria officinalis* (4.36), *Tussilago farfara* (5.65), and *Picrorhiza kurroa* (8.20). For *Helichrysum italicum*, the best multiplication rate (16.4) was obtained with only one immersion per day (i.e., 5 min/24 h), considering that a notable decline was found during the immersion of 1 or 5 min every 8 h.

A longer immersion time of 3 h was efficient to proliferate *Pistacia lentiscus*, a species with difficult propagation, either by seed or by cuttings [88], whereas shortest times of only 10 s succeed to micropropagate *Stevia rebaudiana* through a TIS bioreactor; in particular, maximum values of number of shoots per explant (8.47) were obtained with immersion for 10 s/1 h [97].

#### 3.3.2. Immersion Frequency and Bioactive Compounds Content

As reported above, the significant effect of the immersion cycle on biomass accumulation was evidenced in medicinal plants, and thus it is important to highlight its impact on the large-scale production of bioactive compounds as well (Table 2).

*Castilleja tenuiflora* shoots from an immersion cycle of 30 min every 24 h showed the highest total phenolics (30.58 mg GAE/g DW) and total flavonoid content (45.83 µg CE/g DW) [118], while a lower immersion frequency of 15 min every 12 h increased only the flavonoid (32.00 mg catechin equivalent-CE/g DW) in shoot culture of *Gynura procumbens* [125]. In *Rosa canina* plantlets, two immersions per day (15 min/12 h) led to larger content of phenolic compounds (15.8 mg/g FW), whereas more immersions (15 min/6 h) produced a higher accumulation of soluble sugars (9.2 mg/g FW; [132]). On the other hand, both betalains (betacyanins and betaxanthins) in hairy root culture of *Beta vulgaris* [117] and saponin in microtubers from *Chlorophytum borivilianum* [121] increased at 15-min flooding every 1 h during the cultivation in RITA.

As cardiotonic glycosides (digitoxin, digoxin, and lanatoside C) are secondary metabolites of high value for the pharmaceutical industry, it was interesting to enhance their net production in *Digitalis purpurea* shoots cultured in TIS at six immersions per day (2 min/4 h) [123]. Valuable Amaryllidaceae-type alkaloids, such as galanthamine, its precursor norgalanthamine, and lycorine, were gained from *Leucojum aestivum* with 15 min/8 h as the immersion cycle [127]. In *Pancratium maritimum* shoots, the biosynthesis of alkaloid was affected by the immersion frequency. An immersion frequency with 15 min/12 h increased the content of total intracellular alkaloid by 58% and 54% as compared to 15 min/6 h and 15 min/24 h, respectively [131]. The immersion of 10 min/80 min was effective to optimize the accumulation of total phenolic acid, total phenylethanoid, and total phenol contents in shoots of *Salvia viridis* [134].

### *3.4. Influence of TIS on Medium Composition*

The content of the nutrient solution used in TIS cultures plays a key role in plant growth. It should be also noted that, in some cases, the liquid culture in TIS can contribute to the use of a lower number of mineral salts and PGRs in comparison to the conventional culture in semi-solid medium. Indeed, the use of liquid media, in addition to reducing the production costs by eliminating the gelling agent [152], contributes to a more efficient use of the culture medium, as the explants are in direct contact with the liquid medium during the immersion period and can absorb the nutrients more effectively [26,30,153].

#### 3.4.1. Medium Composition and Biomass Production

In *Myrtus communis*, the propagation in PlantForm (Figure 4) at an immersion cycle of 8 min/16 h allowed the reduction of the macro- and micronutrients concentration in the

liquid medium by half without compromising their growth rates, and therefore greatly reduced propagation costs [82].


**Figure 4.** Micropropagation of *Myrtus communis* in semi-solid medium and in PlantForm bioreactor (**a**); shoots at the beginning of culture in PlantForm (**b**); shoots after 8 weeks of culture in PlantForm (**c**); plants after 2 weeks (**d**), 4 weeks (**e**), and 4 months (**f**) of ex vitro acclimatization (Photo by De Carlo A).

The effect of the PGRs present in the liquid culture medium is more efficient in the TIS system, as demonstrated by Benelli and De Carlo [84], for the micropropagation of olive trees using PlantForm with a cycle of 8 min/16 h. Despite the stringent hormonal needs of the in vitro olive tree, it was possible to reduce the concentration of zeatin by half; there were no significant differences in shoot length when 5 or 10 µM zeatin was applied (3.04 and 3.13 cm, respectively). The need for this expensive hormone for the propagation of the olive tree is well known to obtain an efficient multiplication rate in this species.

Some authors have monitored the effect of different sucrose concentrations in the culture substrate on biomass production. In particular, *Curcuma zedoaria*, *Zingiber zerumbet*, *Gynura procumbens*, and *Bletilla striata* have showed that a higher concentration of sugar improves the performance of explants [52,62,73], whereas a significant increase in shoots number (13.05) of *Bambusa vulgaris* was noted in TIS with a sucrose-free medium [51].

The growth of *Aristotelia chilensis* in a two-vessels bioreactor under optimized photomixotrophic conditions using reduced concentration of sucrose (1% and 2%) and air-supplemented with $CO_2$ (0.4 MPa) did not differ significantly in biomass production (FW per cluster of plants) compared to the control treatment with sucrose 3% and standard air [49].

Rosales et al. [96] tested the efficacy of five substrates for the cultivation of *Stevia rebaudiana* in TIS (BIT bioreactor): different concentrations of sucrose (30 or 40 g/L), presence or absence of kinetin (1 mg/L), and a microbiological suppressant (1 mL/L of methylisothiazolone and methylchloroisothiazolinone) were added to the basal medium. In a culture medium containing 30 g/L sucrose and microbiological suppressant, the best results were observed during ex vitro acclimatization, reporting survival in 92% of plants. With *Anthurium andraeanum* cultured in RITA at an immersion cycle of 3 min/3 h, the presence of 6-benzylaminopurine (BA) alone increased the multiplication rate better than the combination of BA with the other hormones (indole-3-acetic acid, gibberellic acid) [45].

#### 3.4.2. Medium Composition and Bioactive Compounds Content

The content of total phenolic compounds, as well as phenylethanoid glycosides verbascoside and isoverbascoside, were increased in *Castilleja tenuiflora* shoots exposed to modified B5 culture medium [154] containing less nitrogen amounts using the RITA system at 5 min/24 h [119].

The combination of elicitor (Chitoplant®) and TIS (two vessels bioreactor), as a reliable alternative for the genetic modification, increased the cardenolides production 2.2-fold in *Digitalis lanata* shoots [122], significantly improved galanthamine content in the leaves and bulblets of *Leucojum aestivum* [126], and stimulated the production of thapsigargin and nortrilobolide in vitro *Thapsia garganica* shoots [137]. Moreover, the elicitation induced the production and accumulation of phenylpropanoid metabolites in both fresh biomass and culture medium of *Aristotelia chilensis* [49].

Since the concentration and the type of PGRs are often considered a crucial factor for the accumulation of secondary metabolites, Langhansova et al. [129] found that medium containing benzo[b]selenienyl acetic acid (BSAA) and kinetin was the most suitable for a large-scale production of saponin in *Panax ginseng* cultured in TIS (RITA bioreactor).

## 4. Conclusions and Perspectives

The production of secondary metabolites of industrial interest (for health, cosmetics, and food use) is frequently based on the exploitation of natural plant resources through the collection of endemic species not subject to specialized cultivation, thus compromising their genetic resources. When these substances are found in cultivated species, the extraction rates are often so limited that are not economically advantageous. For this reason, it is of strategic importance to search for alternative ways of obtaining these substances in large quantities. The modern biotechnology offered today provides important tools for the production of biomass and bioactive compounds, consistently limiting the exploitation of natural resources. The micropropagation in bioreactors using liquid media has significant potential for commercial purposes, in particular with the application of the liquid culture in TIS, where the brief contact times of the plant material with the liquid medium allows it to limit its decay while maintaining high biomass production values. Since the first report of TIS application on medicinal plants, much research has been carried out and progress in science and technology has made a great step forward, as highlighted in this review.

The research highlights the need to continue developing and optimizing effective liquid culture protocols in TIS bioreactors in order to enhance, as much as possible, the

production of biomass, the number of subculture cycles, and the concentration in plant tissues of bioactive compounds of health interest. Moreover, ease in the operation and handling of the devices and improvement of the automation are desirable, so as to expand the possibility to apply the system on a large scale with decrease in the production costs at a commercial level. Through a literature review, the present paper argues that TIS culture can be a strategy to enhance the supply and quality of plants used for medicinal purposes. For example, in *Curcuma longa* the objective was to develop and refine the in vitro multiplication system for mass propagation by TIS, thus to help restore the fragile ecosystem on Rapa Nui, given that the potential overexploitation of this medicinal plant raises doubts about its long-term survival in this area [61]. The advances in TIS culture represent a way which will enable rapid multiplication and sustainable use of medicinal plants for future generations, contributing to meeting the social, economic, and commercial demands, but with the awareness to preserve plants as essential natural resources.

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