

Review Article

Callus Culture for the Production of Therapeutic Compounds

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Abstract: Plant-derived compounds retain a special place in the treatment of various diseases across the world. Their application cuts across every class of disease, where they are found to be often equal or of greater potency, safer and cheaper than so-called "orthodox" medicines. These advantages have led to great interest in the use of callus culture as a biotechnological tool for the harnessing of these useful therapeutic compounds. Callus culture techniques aim to increase the yield of active constituents in cultured plant cells and to produce novel products on a large scale. These techniques have been applied to produce various classes of therapeutic compounds from diverse plant species through empirical determination of ideal culture conditions and other methods. This review presents at a glance the recent advances being made in the field of callus culture for the production of therapeutic compounds, with the aim of showing that it is time for the full potentials of callus culture to be exploited on a scale that will prove a useful weapon in the arsenal of clinical therapeutics.

Keywords: Callus Culture, Plant Growth Regulator, Elicitor, Precursor, Anticancer, Antiviral, Antioxidant, Therapeutic Nanoparticles

1. Introduction

Callus culture is crucial in the use of biotechnology to harness plant products for man's benefit. It involves the growth of plant tissues into an undifferentiated mass, which can then be engineered to yield useful products for pharmaceutical, food, agriculture, and cosmetic industries [1]. The development of callus culture as a biological engineering process for therapeutic compounds, so-called pharmaceutical engineering, has gained much prominence in recent years, employing various techniques to maximize the production of useful compounds by specific plant cells [2].

The products obtainable from plant cells are generally divided into two classes, namely primary and secondary

metabolites. The primary metabolites are essential for the survival of the plant cells while secondary metabolites are not needed by the plant, but are produced in response to injury, or for protection against various kinds of biotic and abiotic stress [3]. It is therefore a convenient happenstance that most of these secondary metabolites which include alkaloids, tannins, glycosides, terpenoids and flavonoids, have therapeutic uses for disease management in humans [4].

In callus culture for production of therapeutic compounds, at least two steps are generally required; the first for maximum callus growth, and the second for production of secondary metabolites [5]. For callus formation, optimum conditions of light, temperature, humidity and nutrients are required. Calli are formed due to different biotic and abiotic stimuli. The

foremost plant physiologist FW Skoog experimentally demonstrated that intermediate ratios of auxins and cytokinins are generally responsible for callus induction *in vitro* [6]. The second stage involves application of empirically determined quantities of elicitors or precursors to maximize the production of target secondary metabolites [7]. There are two main types of elicitors; biotic (pectin, pectic acid, chitin, chitosan, glucans) and abiotic (extremes of temperature, ultraviolet light, osmotic pressure, antibiotics, fungicides, salts), which act by inducing the transcription of relevant parts of the plant genome to produce secondary metabolites [8].

This review aims at presenting recent thrusts in the use of callus culture for production of anticancer, antibiotic, antimalarial, antifungal, antiviral, nanoparticles and antioxidant compounds.

2. Callus Culture as Means of Therapeutic Compounds Production

Callus culture is a faster and more reliable means of obtaining therapeutic metabolites compared to collection of plant materials from the wild [5]. It is also good for conservation of endangered species, since large amounts of plant material for industrial-scale production do not have to be collected [9].

Callus culture presents great advantages due to its being amenable to industrial production [10]. The metabolites produced from callus culture are very diverse and not restricted to any class of compounds [11] being found commonly in use for treatment of almost all ailments across the world. It is therefore an important step to first identify which plant species has the capacity to produce the compound of interest and most companies have a database of lead compounds produced by various species [12].

The industrial process of therapeutic compounds production from callus cells revolves around the choice of the right bioreactor for the fermentation process, optimal temperature and pH as well as providing the right amounts of nutrients to suit the cultured plant [13]. Normally, callus cultures are converted into suspension culture systems, which employ either batch or continuous fermentation processes that yield.

3. Callus Culture for Specific Therapeutic Compounds

3.1. Callus Culture for Anticancer Compounds

According to the World Health Organization, cancer is the second leading cause of death worldwide as at 2018 [14]. About 1 in 6 deaths are due to cancer, with over 70% occurring in low-middle income countries [15]. Cancer's total cost to the global economy was estimated in 2010 to be approximately 1.16 trillion USD, while the total healthcare costs in the US alone in 2015 was estimated at 80.2 billion USD [16].

The high mortality rate of cancer in low-middle income countries is a reflection of how costly cancer treatment is, and

as a result, new ways of producing cheaper anticancer medication are being explored. Since a wide range of the most important anticancer drugs are of plant origin, tissue culture of such plants have been employed through various techniques to improve the supply and thereby drive down the cost of anticancer drugs [17]. There are several compounds derived from plants for the treatment of cancer such as Paclitaxel (Taxol), Camptothecin, Vincristine, Vinblastine, Vinorelbine, Vindesine, Pomiferin, Sulphoraphane, Noscapine, Epipodophyllotoxin, among many others.

3.1.1. Paclitaxel (Taxol)

Paclitaxel is a major compound in the management of cancer worldwide. It has been called the best-known anticancer agent derived from natural products [18]. It is widely considered as first line in the treatment of breast, ovarian and some lung cancers, among other types [19]. As a result of its wide usage, global demand far exceeds current supply [20], and tissue culture has been employed to increase supply of the molecule since the 1990s.

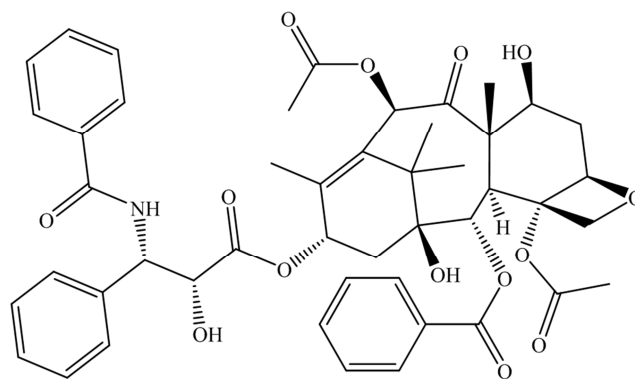


Figure 1. Paclitaxel (Taxol).

Recent advances for tissue culture production of paclitaxel are centered around culture media optimization, precursor feeding and the use of elicitors.

Elicitors are good for secondary metabolite production, but they tend to inhibit callus growth, hence necessitating the empirical determination of the right elicitor concentration for different species [21]. Sarmadi *et al* (2019) recently found that 2-3% concentrations of Polyethylene glycol acted both to improve viability and also increase by more than double the paclitaxel yield of *Taxus baccata* callus culture [22]. Similarly, Yamamoto *et al* (2014) found that 0.76-7.60 μM of 5-aminolevulinic acid promotes both callus growth and paclitaxel production in *Taxus caspidata* cultures [23]. However, higher concentrations had negative effects.

In a robust study to determine the simultaneous effects of elicitors, precursors and metabolic inhibitors on paclitaxel production by *T. caspidata* cell cultures, Wang *et al* (2016) showed that the optimal concentrations of methyl jasmonate, salicylic acid, phenylalanine and gibberic acid were 100 μmol , 20mg/L, 400mg/L and 2mg/L respectively, leading to an over 10-fold increase in paclitaxel production [24]. This shows that optimizing paclitaxel production requires a multifaceted approach.

3.1.2. Camptothecin

Camptothecin was discovered in 1966, and is highly effective against solid tumors, breast, lung and colorectal cancers [25]. Annual demand is estimated at 3000kg/year whereas global annual production does not exceed 600kg [26]. This means at least a five-fold increase in production is required.

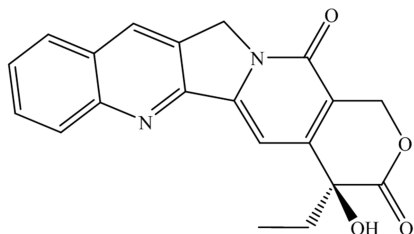


Figure 2. Camptothecin.

Early efforts at producing Camptothecin (CPT) by cell suspension culture of its primary source, *Camptotheca acuminata*, showed unfavorable results [27], and similar attempts for callus and cell suspension cultures of another well-known CPT source, *Nathophodytes foetida*, also exhibited suboptimal production of CPT [28].

However, the use of elicitors has shown more favorable results. For instance, *Nathophodytes nimoniana* callus cultured on solid MS media were elicited with yeast extract and vanadyl sulphate leading to an overall increase in CPT production with vanadium sulphate also showing positive effects on callus biomass [29].

The provision of optimal growth conditions for callus culture can in and of itself lead to an increased yield of secondary metabolites as found by Krishan *et al* (2018) that unelicited callus and suspension cultures of a herbaceous plant, *Ophiorrhiza mungos* had twice the amount of CPT in native plants [30]. A similar study carried out by Thriveni *et al* (2015) showed that CPT was obtained in unelicited calli of *Miquelia dentate*, but amounts obtained were less than that of the native plant [31]. This shows that different species behave uniquely to the presence/absence of elicitors.

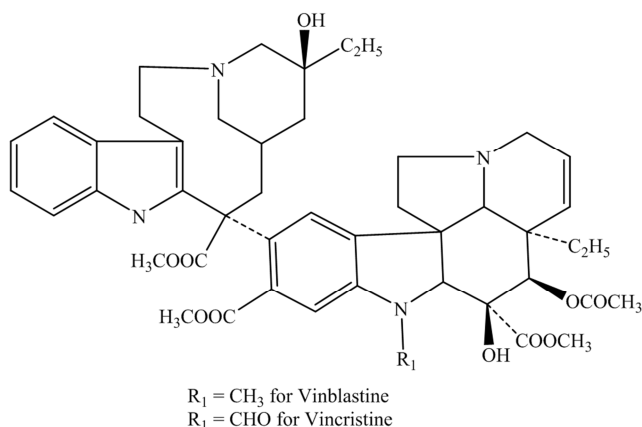


Figure 3. Vinblastine and Vincristine.

3.1.3. Vinca Alkaloids

The vinca alkaloids, of which vinblastine and vincristine are the major compounds, are found in the plant *Catharanthus*

roseus, making the plant somewhat of a celebrity in the medicinal plants community. They are used as treatment of choice for leukemia, Hodgkins/Non-Hodgkins lymphoma, breast, testicular and germ cell tumors [32].

However, the very low yield of the alkaloid-500kg is required to produce 1g of vinblastine- makes them rather expensive and often unavailable [33].

Kaur *et al* (2017) demonstrated the effect of various plant growth regulator concentrations on the levels of vinca alkaloids production in calli of *C. roseus*, where they found that abscissic acid and gibberic acid negatively affected alkaloid production, while ethephon, chlormequat, salicylic acid, 2,4-D, NAA and triadimefon increased vinblastine levels [34]. Zuhairi and Obaid (2017) also found similar negative effects of abscissic acid on vinblastine production in *C. roseus* calli [35]. Attempts to increase yield by both biotic and abiotic elicitors have also been done, with biotic elicitors (chitosan, yeast extract) enhancing secondary metabolite production by up to 48% [36, 37].

Several novel anticancer agents are also being discovered through callus culture. Pandey *et al* (2015) were able to obtain high yields of the novel anticancer compound betulinic acid from callus cultures of different species of *Ocimum* through elicitation with Methyl Jasmonate. However, all native *Ocimum* species tested showed no betulinic acid synthesizing capacity, except *Ocimum kilindscharium* [38]. This shows that the production of betulinic acid in this experiment was a case of novel molecule appearance in calli, as is often the case in callus culture [39]. A similar discovery was made by Jan *et al* (2017) that a novel compound, salviolactamine, effective against cervical and prostate cancer, was found in calli of *Salvia santalinifolia*, whereas the compound is absent in the native plant [40].

3.2. Callus Culture for the Production of Antibiotics

Antibiotics have originally been natural products until the industrial revolution when chemical synthesis took preeminence [41]. With the ever-rising scourge of antibiotic resistance leading us to the brink of what some scientists have called an impending 'antibiotic apocalypse', attention has largely returned to natural products as the source of new compounds that will prove effective and safe, and fight resistant strains of microorganisms [42].

Arias *et al* (2018) found that the callus culture of *Thevetia peruviana* showed high antimicrobial activity against both gram negative and gram positive bacteria. The assay was carried out using crude extracts, and as is usually the case, the extract with one solvent performed better than others- the hexane extract of cell suspension culture performed better than the callus culture, while the ethanol extract of the callus culture did better than that of suspension culture [43].

In another study, Al-saleh *et al* (2019) investigated the in-vitro grown shoots versus calli of *Ammi visnaga* for antimicrobial activity. They found that extracts of both in vitro microshoots and calli had better antimicrobial activity than the native plant [44].

While many studies focus on the biological activity of crude

extracts, there are those who target production of specific compounds known to have anti-infective property. For example, Choes-Guaranda *et al* (2019) focused on the production of lupeol from the calli of *Venonanthura patens* [45] while Rameshkumar *et al* (2018) focused on squalene production in callus cultures of *Nilgiranthus ciliatus* [46]. A wide variety of compounds from flavonoid, alkaloid, glycoside and other families show antibacterial property which is often comparable or even superior to commonly used orthodox pharmaceuticals [47].

3.3. Callus Culture for the Production of Antifungals

There has been a steady rise in the cases of fungal diseases worldwide in recent years. An estimated 1 billion people suffer from fungal infections every year, resulting in approximately 1.5 million deaths [48]. Fungal resistance to the available medicines is a major problem contributing to increased morbidity and mortality [49]. It however appears that antifungal medicines are not being given sufficient research attention but tend to be neglected [50], and as a result, most of the literature reports of antifungal assays are carried out as an add-on to other bioactivity assays. Many of the more popular antifungal drugs work by interference with cell wall synthesis or metabolic poisoning [51]. However, due to the similarities between fungal cells and human cells, being both eukaryotic, many effective antifungals tend to be toxic [52]. Plant extracts with wider safety margins can prove very invaluable [53].

Zubricka *et al* (2015) compared the antifungal activity of Xanthenes found in suspension cultures of selected *Hypericum* species with that found in native and hairy roots of the same plant. The native roots showed better activity than cultured tissues, while the chitosan-elicited cells were inactive against fungi (*Candida albicans*). This probably reflects the fact that Xanthenes are predominantly found in the roots of the plant, compared to aerial parts, and the explants were sourced from leaves [54].

Begum and Mirza (2018) undertook comparative antifungal profiling of callus culture extracts of *Cichorium intybus* vis-à-vis a standard antifungal drug, Nystatin. The bioactivity was shown to vary significantly with solvent polarity, with the moderately polar extracts being more active than the more polar ones. This highlights the importance of lipophilicity especially in antifungal drugs that work by cell membrane disruption [55].

3.4. Callus Culture for Antimalarial Compounds

Malaria remains among the leading causes of death and infirmity in the developing world. Annually, over 200 million new cases of malaria are reported (WHO, 2019). This has led to a huge demand for antimalarials medication that conventional production struggles to meet, leading to high prices of these essential medicines [56].

The frontline compound recommended by the WHO for malaria treatment, artemisinin and its derivatives, have been produced in callus as well as suspension culture, as it is naturally very low in the natural plant [57] hence the effect of

elicitors on improved artemisinin production has also been extensively studied. In a study by Yuliani *et al* (2019), *Aspergillus spp* native to *A. annua* was used as an elicitor, resulting in an over 7-fold increase in artemisinin content [58]. This massive increase occurred with no adverse effect on callus mass, which is a common tradeoff with abiotic elicitors [59]. Abiotic elicitors used include cobalt nanoparticles [60], methyl jasmonate, chitosan, salicylic acid, all with increased yield of artemisinin [61].

Tahir *et al* (2016) showed that an ideal plant growth hormone combination for callus formation in *Artemisia annua* was 0.5µM/L of both 6-benzylaminopurine and Naphthalene Acetic acid [62], similar to results obtained by Yuliani *et al* (2018) [58] although similar results were obtained by other workers with higher concentrations of BAP and different auxins [63].

Another important antimalarial is quinine, although it has lost favor due to its high toxicity, and availability of better alternatives, it is reserved for malaria in pregnancy and as an important second line treatment where Artemisinin-based therapies are unavailable [64]. One source of quinine, *Cinchona ledgeriana*, has been grown as callus culture with application of different chemical elicitors, which had negative impact on cell growth, but increased the production of quinine alkaloids [65].

3.5. Callus Culture for Antivirals

Viral infections present some of the greatest health threats to humanity. From more well-known viruses like HIV and Hepatitis viruses, to emerging ones like Ebola and Zika viruses, millions of are lost globally every year. Viruses present a peculiar challenge to pharmacotherapy because of their tendency to mutate easily and become resistant to current drugs R. The search for antiviral medicines among plant life has produced some important results from plants like *Phyllanthus spp*, *Sylibium marinum*, and a slew of others that show good antiviral activity and are already being commercialized [66].

An efficient callus induction protocol has been developed for *Phyllanthus debilis*, comparing callus induction from leaf and nodal explants. The leaf explants showed better hormone-dependent callogenic properties, but when hormone concentrations were lowered, intermodal explants showed better callusing. This shows that the response to callus formation is determined by the plant part used as explants, as well as the cytokinin/auxin balance [67].

Furthermore, the effect of different wavelengths of monochromatic light as elicitors of secondary metabolism by *S. marianum* was studied by Younas *et al* (2018), where they found that the level of the antiviral compound, sylimarin, was twice that of the control. This is a useful discovery because the light used in tissue culture rooms for sylimarin can be filtered to supply only the red region of the spectra for maximum yield [68].

3.6. Callus Culture for Production of Therapeutic Antibodies

Antibodies have a wide range of therapeutic and diagnostic

applications ranging from cancer management to vaccines and infectious disease treatment [69]. In the past, antibodies were derived from animals, but with the advent of genetic engineering, microorganisms and plants are being used [70]. Plants present the advantage of being fast, safer, and more economic in producing useful antibodies [70].

Transformed callus cultures are produced mainly using *Agrobacterium*-mediated transfer of the gene encoding the needed antibody [69]. The most widely used plant-based systems are tobacco and rice suspension cultures because they are well studied and also amenable to fermentation [71, 72].

Bevacizumab, a monoclonal antibody used in management of colon cancer, lung cancer, glioblastoma and renal-cell carcinoma, has been produced from transgenic callus cultures of rice R. Expression levels of the antibody was reportedly higher than that obtained from previously used expression systems. Using ELISA, it was shown to have similar binding affinity to its antigen as a commercially obtained brand, Avastin® produced from animal cell culture [73].

Callus cultures of *Daucus carota* have also been employed to produce a potential oral vaccine against atherosclerosis. *Agrobacterium*-mediated transfer of the gene encoding Apo B100-a self antigen responsible for atherosclerosis, was employed. The results showed appreciable expression levels, and an immunoblot analysis demonstrated antigenicity of the carrot-made protein. Oral immunization of mice with the proteins also showed good immunogenic activity against the atherosclerosis marker. This could become a good way of delivering the vaccines orally with edible carrots, thereby eliminating tedious downstream procedures for purification [74].

Furthermore, a study by Hidalgo *et al* (2017) on the production of recombinant tissue plasminogen activator protein (a heart attack medicine) in tobacco calli which were transformed and made into suspension cultures, showed appreciable expression of 0.277% after a two-week growth period. However, this level of expression is not high enough to rival the current commercial production using *Escherichia coli* [75].

3.7. Callus Culture for Silver and Related Nanoparticles

Silver nanoparticles (NPs) in the size range 1-100nm have become well known for their wide range of potential therapeutic uses as antibacterial, antifungal, antiviral, antidiabetic, anticancer treatment options [76]. The attractiveness of these NPs stems from their application and smart delivery which reduces side effects and improves therapeutic outcomes [77].

Therapeutic NPs can be made through physical, chemical and biological means, but the biological method employing bacteria, fungi and plants is receiving more attention due to safety and ecological concerns with the other methods [76].

Callus culture has been used to produce biogenic silver NPs which showed antifungal activity against *Candida* species R. The callus was obtained from leaves of *Gymnema sylvestre* induced with 2,4-D and kinetin. The callus extracts efficiently bioreduced the silver nitrate feed producing nanoparticles,

which were shown to be as effective as the antifungal drug Voriconazole [78].

Since secondary metabolites in plant cells are responsible for nanoparticle production, the reductive potential of various plant cells will vary based on relative catalytic capacity. Shkryl *et al* (2018) demonstrated this point by showing that *Nicotiana tabacum* callus cultures transformed with a silicatein gene showed three times the bioreduction capacity of untransformed ones. Silicatein is a hydrolase from marine sponge and was likely responsible for the increased bioreduction capacity. The synthesized silver NPs showed strong antibacterial activity against *E. coli* and *Agrobacterium rhizogenes* [79].

Iyer and Panda (2018) also synthesized gold and silver NPs from pumpkin callus cultures, and further demonstrated the antibacterial efficacy of the silver NPs against *E. coli* DH5 α [80].

3.8. Callus Culture for the Production of Antioxidant Compounds

Oxidative stress is well reputed as being at the root of several disease conditions including cardiovascular disease, diabetes, Alzheimer's disease, Parkinsonism, Cancer, increased susceptibility to infections, and aging [81]. As a result, antioxidants have gained a steady relevance in the management and prevention of several of these diseases. Both synthetic and naturally-sourced antioxidants are widely in use. Common natural antioxidants include vitamins C and E, Polyphenolic, Carotenoid and Flavonoid compounds obtained from plants. The natural antioxidants market stands at about 1 billion USD in the USA alone, with a projected increase by up to one-fifths in the next three years [82].

Presently, antioxidants such as Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT), propyl gallates and EDTA are produced synthetically [83], but increasing concern about safety is driving interest in naturally obtained compounds especially from plants [84].

Callus cultures of *Plectranthus barbatus*- a widely used plant for food and healing- were induced with varying concentrations of hormones on MS media, and active compounds were extracted from harvested calli using methanol and hexane. The extracts were then tested for antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Results showed that the more polar methanol extract showed highest antioxidant activity, with the callus extract being more effective than the in-vitro grown plant extract [85].

Furthermore, the effect of various elicitors has been investigated in callus cultures of various species. Sarmadi *et al* (2019) showed significant increases in antioxidant expression when Polyethylene Glycol (PEG) was added to callus cultures of *Taxus baccata*. Similarly, Gamma irradiation of *Artemisia annua* callus cultures led to marked increases in total phenol and flavonoid content, which correlated to higher antioxidant activity (measured by DPPH scavenging potency) in the irradiated callus cultures from control cultures. The effect was seen to be dose-dependent, with 15Gy radiation produced best

results compared to other doses from 5-35Gy [22].

In addition, the effect of light wavelengths on the expression of phenolic and flavonoid antioxidants in *Stevia rebaudiana* was also demonstrated by Ahmad *et al* (2015), where blue light showed highest phenolic and flavonoid accumulation, while red lights increased free radical-scavenging activity. The increase in total phenols and flavonoids correlated in direct proportion to increased antioxidant property in blue light-treated callus cultures, while such correlation was weak in the case of red light-treated cultures, suggesting that other compounds apart from flavonoids and phenols may be responsible for their increased free radical-scavenging activity [86].

Silver and gold NPs have also been shown to elicit increase in phenol and flavonoid content as well as corresponding increase in antioxidant property. The observations were made in callus cultures of *Prunella vulgaris* induced by Naphthalene Acetic Acid (NAA) in combination with the NPs. The results showed that NPs increased antioxidant activity in callus cultures even in the absence of concomitant NAA [87].

4. Discussion

There is considerable interest in callus culture as a means of producing therapeutic compounds. The literature shows that callus culture of plant cells serves as bioreactors that are safe and economical for the production of therapeutic compounds.

It can be seen that the use of callus culture for anticancer compounds production is the most advanced. This may be attributable to government policies that target funding towards cancer research, as well as the high market value of anticancer compounds. As a result, specific compounds are usually the target of callus culture, since their biosynthetic pathways are known, paving the way for the use of precursors and specific elicitors.

On the other hand, unlike the case of anticancer compounds, callus culture for antibiotic and antifungal compounds does not tend to narrow down to specific target compounds. Rather, crude extracts are often tested for therapeutic activity, which is attributed to broad classes such as the presence of alkaloids, saponins, and tannins. Hence, a more general approach is usually taken for improvement of therapeutic activity of such callus cultures.

For antimalarials and antiviral compounds, specific compounds have been identified and targeted. This trend shows a possible correlation between the severity of diseases in terms of morbidity and mortality and the likelihood of callus culture for specific compounds targeting it. The availability of effective alternatives (e.g. antibiotics) also seems to decrease interest in callus culture for specific target compounds.

As for the use of callus culture for the production of nanosilver and other NPs, very specific reactions are involved (bio-reduction), and callus culture serves mainly to produce metabolites that facilitate the reaction. The mechanisms are still poorly understood, but with continued research in this area, much will be uncovered soon.

5. Future Prospects

At present, the advancement of callus culture as an economically viable means of producing therapeutic compounds is slowed due to the difficulty of scaling up production to industrial scale and also prohibitive costs of downstream processing. Downstream processing alone is said to be responsible for 80% of total cost of production of therapeutics from callus culture [88]. However, with continuous advances in computer-aided modeling, coupled with better understanding and engineering of the production process, callus culture will eventually gain much deserved prominence in the field of therapeutics production.

Furthermore, the application of callus culture for production of novel therapeutics will continue to receive attention as the search for new remedies for diseases that have become resistant to current treatment continues. Antibiotic resistance, cancer, and emerging diseases will remain major drivers in this direction.

Research is also likely to focus more on transgenic callus culture and synthetic biology as the genetic basis of the therapeutic capability of useful plant species becomes well elucidated. The increasing acceptance of genetically modified organisms will help encourage this.

The preservation of endangered plant species will also continue to be a factor in the encouragement of callus culture. As the world's population is projected to reach almost 10 billion by 2050 [89], enormous pressure will be exerted on plants as sources of medicines, and this will greatly endanger several species. Callus culture then will become an inevitable alternative source of therapeutic compounds.

6. Conclusion

Callus culture has been extensively used experimentally and in some cases commercially, to produce useful therapeutic compounds. Such compounds range from antibiotics for treatment of resistant infections, to well known anticancer compounds and medicinal nanoparticles. It is therefore important that as much attention is being given to upstream processes, research into the optimization of downstream processes for easy and cost-effective extraction of callus culture products should receive commensurate attention.

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