Perspective

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Towards dsRNA-integrated protection of medical *Cannabis* crops: considering human safety, recent- and developing RNAi methods, and research inroads

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Abstract

Owing to the expanding industry of medical *Cannabis*, we discuss recent milestones in RNA interference (RNAi)-based crop protection research and development that are transferable to medical *Cannabis* cultivation. Recent and prospective increases in pest pressure in both indoor and outdoor *Cannabis* production systems, and the need for effective nonchemical pest control technologies (particularly crucial in the context of cultivating plants for medical purposes), are discussed. We support the idea that developing RNAi tactics towards protection of medical *Cannabis* could play a major role in maximizing success in this continuously expanding industry. However, there remain critical knowledge gaps, especially with regard to RNA pesticide biosafety from a human toxicological viewpoint, as a result of the medical context of *Cannabis* product use. Furthermore, efforts are needed to optimize transformation and micropropagation of *Cannabis* plants, examine cutting edge RNAi techniques for various *Cannabis*–pest scenarios, and investigate the combined application of RNAi- and biological control tactics in medical *Cannabis* cultivation.

Keywords: medicinal plants; RNA; biopesticide; pest management; disease resistance; pesticide safety

1 PROTECTING AN EXPANDING INDUSTRY

Medicines represent an indispensable pillar of society, and remain an area of growing interest with regard to their source-plants and how they are cultivated. *Cannabis sativa* L. and *Cannabis indica* Lam. represent important sources of medicines for human populations. Two key phytocannabinoid compounds of medicinal interest found in *Cannabis* plants include Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), both of which are used to prevent and alleviate a variety of ailments.[1] Pain relief is the most commonly cited reason for medical use of *Cannabis*.[2–5] Considerable evidence also suggests that cannabinoids exert anticancer effects at multiple levels of tumour progression,[6,7] although Xiong *et al*.[8] recently revealed a mechanism suggesting that *Cannabis* and cannabinoids should be avoided during immunotherapy. There also is a growing body of evidence supporting *Cannabis* and cannabinoids for treating patients suffering from drug addiction,[9] multiple sclerosis,[10] anorexia nervosa,[11] epilepsy[12] and Tourette syndrome.[13] We state here, however, that maternal use of *Cannabis* during pregnancy has been associated with higher incidence of autism spectrum disorder in the offspring,[14] an important point given the common perception that it is safe to use *Cannabis* during pregnancy.[15] Aside from clinical use, THC also is widely used globally for its psychoactive effects. Medicinal and recreational *Cannabis* products on the market collectively include those applied via inhalation, ingestion and dermal application, the most widely practiced being the inhalation of combusted flowers.[16] So far, a total of 44 countries and 37 states in the USA, have legalized medical use of *Cannabis*. Six countries, 19 US states and Australia's Capital Territory have legalized recreational use of cannabis.

Cultivation of medical *Cannabis* is a competitive, diverse, rapidly evolving, global industry, and *Cannabis* is a crop of high value (New Frontier Data, https://newfrontierdata.com/cannabis-insights/comparative-yield-per-acre-for-grains-and-

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marijuana; NORML, https://norml.org/news/2022/01/13/analysis-adult-use-marijuana-sales-yield-over-10-billion-in-new-tax-revenue), the largest market being in Canada and the US.[16] This expansion of *Cannabis* production has led to and is expected to continue to lead to increases in pest pressure in both indoor and outdoor *Cannabis* cropping systems, including the detection of regionally novel pest infestations.[17–19] Management of fungal pathogens relies on preventing infection at the earliest stages of *Cannabis* cultivation, and minimizing the spread of spores. Fungi infecting pre- and post-harvest inflorescence tissues are likely to be the most damaging to the medical *Cannabis* industry, especially those of the genera *Botrytis*, *Fusarium*, *Penicillium* and *Golovinomyces*.[20–22] In indoor cultivation, pathogens can easily spread via air and recirculated water; in outdoor cultivation, pathogen spread is more dependent on wind, rainfall and proximity to fields containing alternative hosts.[20] The most serious viral pathogen in indoor *Cannabis* cultivation is *hop latent viroid*, which causes a stunting in growth/development of inflorescences.[20] Chiginsky *et al.*[19] recently assessed the diversity and distribution of *beet curly top virus* affecting outdoor *Cannabis* cultivation in Colorado; given its high incidence and strain diversity, the broad host range of the virus and its vector (the beet leafhopper, *Circulifer tenellus* Baker), and the severity of symptoms, the authors suggest that *beet curly top virus* may become one of the most serious threats to *Cannabis* production. There are several potentially important arthropod pests of medical *Cannabis*, but thus far most efforts to identify these threats have focused on industrial hemp crops, which may differ in susceptibility compared to the stickier, more resinous *Cannabis* strains of medical importance. Cranshaw *et al.*[18] reviewed important leaf, inflorescence and root feeders in the USA, many of which are pests of near-global relevance. Perhaps the most important arthropod pests of indoor medical *Cannabis* cultivation include: cannabis aphid (*Phorodon cannabis* Passerini), rice root aphid (*Rhopalosiphum abdominalis* Sasaki), cotton/melon aphid (*Aphis gossypii* Glover), sweetpotato whitefly (*Bemisia tabaci* Gennadius), onion thrips (*Thrips tabaci* Lindeman), western flower thrips (*Frankliniella occidentalis* Pergande), two-spotted spider mite (*Tetranychus urticae* Koch) and hemp russet mite (*Aculops cannibicola* Farkas).[18,23] Other arthropod pests of increasing consideration for outdoor medical *Cannabis* production include: Japanese beetle (*Popillia japonica* Newman), grasshoppers, brown marmorated stink bug (*Halyomorpha halys* Stål), Eurasian hemp moth (*Grapholita delineana* Walker) and corn earworm (*Helicoverpa zea* Boddie).[17,18,24]

Pest management techniques are key in maintaining desirable crop yields in *Cannabis* production, but herein lies the complexity of producing healthy medicinal *Cannabis* plants in mass quantity. On the one hand, *Cannabis* pests need to be managed effectively in order to produce the downstream medicinal product in a way that is economically beneficial to the producer. On the other, the use of conventional pesticides can be detrimental to the medicinal context of the production of such crops, given the growing evidence of harmful effects of pesticide residues on vertebrates including humans.[25–28] Indeed, pesticide residue contamination in medical and recreational *Cannabis* is an area of ongoing discussion and analysis,[28–31] pesticides having been detected even in medical *Cannabis* samples.[32] Recently, Pinkhasova *et al.*[28] used data from the publicly available Comparative Toxicogenomics Database (CTD)[33] to examine the potential human hazards of pesticide contamination on users of medical *Cannabis*. In a network analysis, the authors explored interactions between pesticide residues, cannabinoids and epilepsy, across key biological functions related to seizures. This study suggests that simultaneous exposure to some pesticide groups and cannabinoids could disrupt shared biological pathways implicated in seizures, epilepsy and other neurotoxic effects. The abovementioned study also highlights that some pesticide groups could individually, or additively, produce neurotoxic effects by interacting through shared mechanisms. The use of conventional pesticides in medical *Cannabis* cultivation may thus increase the risk of dangerous phenotypes in patients using *Cannabis* for medical purposes, especially patients with genetic susceptibilities. Certainly, mass cultivation of plants to be used for human medicinal purposes should require the implementation of pest control techniques demonstrated as safe from a human toxicological viewpoint, and not based on a neurotoxic mode-of-action.

Nonchemical pest management approaches often are used for biosafe, sustainable production of medical *Cannabis*. In indoor *Cannabis* cultivation, vaporized sulfur can minimize powdery mildew disease (e.g. from *Golovinomyces* infestation) establishment, and spray applications of potassium bicarbonate or giant knotweed (*Reynoutria sachalinensis* (F. Schmidt) Nakai) extract can reduce powdery mildew development.[20,34] Integrated pest management (IPM) strategies may be further supplemented with biological control via carnivorous arthropods.[35] Cranshaw *et al.*[18] discussed the prevalence of numerous predaceous arthropod taxa in the USA, albeit in outdoor industrial hemp crops; the effects of medical *Cannabis*'s resin-rich trichomes on the activity of natural enemies still requires investigation. Lemay *et al.*[35] discussed potential limitations to successful biological control of *Cannabis* pests. For example, the authors discussed how secondary metabolites (primarily phytocannabinoids and terpenes) produced by *Cannabis* may interact negatively with predators and parasitoids, via biomagnification of these compounds when transported to this higher trophic level. Furthermore, they discussed how *Cannabis* morphology also may affect the functionality of these biological control agents. In particular, glandular and nonglandular trichome structure/density, and the absence of pollen and nectar in medical *Cannabis* plants (all of which are female), both may represent impediments to obtaining vital food resources.[35] Morphological differences between *Cannabis* strains also affect pest pressure; for example, strains that produce loosely packed inflorescences develop less *Botrytis* infection than tightly packed inflorescences, improved air flow being the assumed reason.[20] Britt *et al.*[17] discussed several biorational options that may be useful in managing *H. zea* in outdoor *Cannabis* crops; for example, *Bacillus thuringiensis* Berliner- and/or nucleopolyhedrovirus-based insecticides. Other potential IPM solutions for *Cannabis* protection, such as determining rotation-crops that minimize pathogen build-up in *Cannabis* crops, and the use of microbial biocontrol agents, also have been discussed recently.[20,36] As biotechnological solutions are advancing, the toolkit for achieving protection of medical *Cannabis* crops, while maintaining an appropriate biosafety profile, is expanding.

2 A BIOSAFE APPROACH IN PRACTICE

RNA interference (RNAi), or double-stranded RNA (dsRNA)-mediated gene silencing, in plant pests is increasingly exploited for IPM efforts. Both transgenic (i.e. RNAi cultivar/strains) and spray-based (dsRNA formulated for topical application to plants) approaches to delivering dsRNA to plant tissues are under adoption and/or consideration on a global scale. A key benefit to

RNA-based pest management is the technology's reported biosafety to nontarget taxa[37–49] (but see Powell *et al.*[50]), deriving from dsRNA's nucleotide sequence-specific mode-of-action on messenger RNA (mRNA) in viruses and eukaryotic cells.[51,52] This sequence-specificity permits the capacity for identifying a wide array of precise molecular targets in a given pest species, allowing triggering of respective phenotypes, including, but not limited to, those potentially most compatible for simultaneously benefiting from biological control services.[53] Although such precise molecular targeting is the cornerstone supporting development of RNA-based pest management practices, the concurrent shaping of a meaningful risk assessment standard for unintended environmental effects of these applications is still in its infancy. The main plausible unintended effects of applied dsRNA include knockdown of important mRNA transcripts in nontarget organisms via direct exposure or other food web associations, and the activation of immunomodulatory responses in nontarget organisms, both of which could result in sublethal or lethal effects in the nontargets.[54,55]

Potentially supportive of biosafety to mammalian taxa, three empirical studies examining nontarget RNAi in the CD-1 mouse model suggest extensive extracellular and cellular barriers to exogenous dsRNA supplied via diet.[37,38,56,57] One of these studies exposed the mice to dsRNA with 100% complementarity to mouse *vacuolar ATPase* (*vATPase*) mRNA, with no observed effects on daily clinical observations, body weight, food consumption, serum biochemistry, haematology, organ weights, and both macroscopic and microscopic pathology.[38] However, a response to this study[58] pointed out that, whereas target mRNA downregulation was confirmed *in vitro* through transfection of siRNA duplexes into mouse kidney cells, the authors of the original study did not test the 218-bpdsRNA in the mouse kidney cell culture. As Petrick *et al.*[38] mentioned the tendency of long dsRNAs to trigger immune responses (and consequently apoptosis) in cultured cells, it is surprising that the collection of such data were not included in the risk assessment. Heinemann *et al.*[58] further pointed out that there was no clarification regarding what phenotypes may arise from targeted downregulation of *vATPase*, and that there was a lack of appropriate endpoint measurement for RNAi risk assessment, as the authors did not analyze vATPase protein expression. Observing stable mRNA levels does not mean that protein levels remain stable; dsRNA may decrease the rate at which proteins are made (translational inhibition) without changing the amount of mRNA; these effects take longer than the study of Petrick *et al.*[38] was designed to evaluate.[58,59] To date, investigating such effects remains still necessary for robustly evidencing mammalian safety with regard to RNAi crop technology, especially when such studies are intended to translate meaningfully to human toxicology and biosafety. Although the pH of stomach fluid in mice and humans is ≈3–4 and 1.5–2, respectively, thus representing a major barrier to dsRNA stability, sprayable dsRNA products (see below) may contain formulants that significantly enhance dsRNA stability. The current and expected additions of formulants to naked dsRNA for sprayable RNA pesticide products further demands robust investigations into the effects of such products in mammalian models.

The first pest-resistant RNAi cultivar of a major crop, papaya (*Carica papaya* L.), was introduced to Hawaii in 1998 to protect papaya crops against papaya ringspot virus,[60] and more crop species have been the focus of pest-resistant RNAi strategies in recent years. For example, the western corn rootworm (*Diabrotica virgifera virgifera* LeConte)-resistant corn (*Zea maize* L.) cultivar MON87411[61–63] is licensed for cultivation in several countries, including the USA, Canada, Brazil and Japan, and has received a safety certificate for import and food/feed use in China.[64] In 2021, Food Standards Australia New Zealand (FSANZ) approved another corn rootworm (*Diabrotica* spp.)-resistant RNAi corn cultivar, DP23211.[64] These cultivars incorporate *in planta* expression of RNAi traits.[64,65] An RNAi cassava cultivar, resistant to cassava brown streak virus and Ugandan cassava brown streak virus, is expected to become available soon for cassava growers in Kenya (Cassava Plus, https://cassavaplus.org/news/kenya-citizentv-gmo-cassava).[66] Lastly, Colorado potato beetle (*Leptinotarsa decemlineata* Say) is the focus of the first sprayable RNA pesticide product; this product, called Ledprona, currently awaits U.S. Environmental Protection Agency registration.[67] Thus, there has been a global acceleration of developments in the RNA pesticide marketplace.[63,66,68]

3 RECENT ACHIEVEMENTS TRANSFERABLE TO RNA-BASED PROTECTION OF MEDICAL *CANNABIS*, AND NEXT STEPS

Other recent developments have accelerated the potential for RNA-based protection of medical *Cannabis*. Several studies have recently developed *Cannabis*-specific protocols for transient gene expression.[69–72] These studies demonstrated stable transformation of *Cannabis* through various *Agrobacterium* infection (agroinfiltration) techniques, collectively reporting high efficiency for exogenous gene expression, and downregulation of endogenous genes, in different *Cannabis* tissues.[69–72] Although these studies did not focus on pest management, assessing, rather, visible reporter phenotypes (e.g. hairy roots) or quantifiable biochemical phenotypes (e.g. cannabinoid biosynthesis), they do offer hope that both transgenic and spray-based RNAi approaches have potential for development in *Cannabis* crop protection. However, Adhikary *et al.*[73] recently reviewed the current status of *Cannabis* tissue culture, noting *Cannabis* species' reputation for being recalcitrant to *in vitro* micropropagation, as well as the limitations and inconsistencies of available protocols. The present authors do assert, though, that experienced *Cannabis* companies generally are understood to have developed tissue culture and micropropagation techniques over the recent decades, but that these are held as trade secrets for competitive advantage within the industry.[73]

Several insect pests of *Cannabis* crops (e.g. aphids, whiteflies, thrips) confer multi-pest pressure through their ability to vector plant viruses within and between crops by use of their piercing/sucking mouthparts. Recent findings indicate potential for managing these vectors and/or their ability to transmit plant viruses through both transgenic and spray-based RNAi approaches.[42,74–76] Notably, Worrall *et al.*[74] observed inhibition of aphid-mediated virus transmission between plants after spraying dsRNA–BioClay (dsRNA-loaded, biodegradable, layered double hydroxide clay nanosheets) targeting a plant virus. More recently, Jain *et al.*[42] demonstrated the capacity of dsRNA–BioClay in protecting sprayed plants from all life stages of the whitefly *B. tabaci*, and Niño-Sánchez *et al.*[77] demonstrated prolonged plant protection against *Botrytis cinerea* Pers., using dsRNA–BioClay spray, compared to naked dsRNA. DsRNA–BioClay spray technology is currently being further developed through a partnership between the agricultural chemical company Nufarm (Melbourne, Australia) and the University of

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Queensland. Another outstanding study demonstrated enhanced stability of target (*F. occidentalis*)-specific dsRNA expression in transplasmotic plants (where the foreign gene is inserted into the DNA of plastids, e.g. chloroplasts, rather than into nuclear DNA), resulting in significantly greater thrips control efficacy on transplasmotic plants compared to nuclear transgenic plants.[76] Although none of the abovementioned studies used *Cannabis* plants as models, these recent findings undoubtedly represent great strides towards RNAi-based plant protection against insect vectors and the plant viruses they can transmit in *Cannabis* cropping systems, given the taxonomic overlap between the abovementioned studies and real-world *Cannabis* infestation scenarios for both indoor and outdoor growing conditions.

DsRNA seed treatment technology also is gaining momentum, especially following the recently formed partnership between the pioneering dsRNA-based crop protection company GreenLight Biosciences (Medford, Massachusetts, US) and the seed treatment technology company Germains Seed Technology (King's Lynn, Norfolk, UK) (GreenLight Biosciences, https://www.prnewswire.com/news-releases/greenlight-biosciences-and-germains-seed-technology-partner-to-explore-development-of-worlds-first-dsrna-seed-treatment-to-control-pests-301471607.html). Other partnerships are likely to form in this regard, given the potential for combining these biotechnologies towards the control of difficult fungal and viral pests. For example, a number of microbial pathogens can be present on *Cannabis* seeds and potentially infect the growing seedling.[19,20,22] For scenarios such as these, dsRNA seed treatments may eventually prove to represent a breakthrough RNAi technique for target-specific management of certain fungal and viral pests of *Cannabis* crops. However, this technique may only prove effective against pests that colonize the crop during early stages of plant development, given the eventual degradation of dsRNA within plant tissues.[78] The various existing RNAi approaches to plant protection (e.g. nuclear- and plastid-mediated RNAi, dsRNA sprays), together with other promising methods in development (e.g. dsRNA seed treatments, potentially effective in controlling seed-associated microbial pathogens), warrant optimism towards harnessing both *in planta* and exogenous RNAi tactics for protection of medical *Cannabis*. However, we note that dsRNA spray applications may eventually prove to be malproductive in controlling fungal pathogens on indoor medical *Cannabis* crops, as most indoor cultivation uses hydroponic techniques,[20] maintaining drier conditions for aboveground *Cannabis* tissues, which helps prevent colonization, spread and prevalence of fungal pathogens. Still, this does not rule out the eventual development of dsRNA spray tactics for controlling fungal pathogens on either indoor or outdoor medical *Cannabis* crops.

Effective or optimal RNAi approaches, and opportunities for utilizing these tactics, will certainly vary depending on the target pest, as well as between medical *Cannabis* cultivation systems. Sensitivity to dsRNA has been shown to vary dramatically, both between and within taxonomic groups.[79,80] With regard to exogenously applied dsRNA, some major hurdles here may be mitigated through the use of nanocarriers,[81,82] engineered microbial carriers,[83] or the co-targeting of specific molecules (e.g. dsRNases).[53] Although many pests of medical *Cannabis* will exhibit different levels of pest pressure between indoor and outdoor cultivation systems, exogenous dsRNA applications (e.g. spraying, seed treatments, hydroponic systems) should be easily tailorable for combating different pest-pressure scenarios in both indoor and outdoor production. Furthermore, the biosafety profile of RNA pesticides may allow stacking of dsRNAs to target multiple pest species simultaneously in medical *Cannabis* production; this stacking approach could be easily implemented in exogenous applications.

Our research group recently discussed potential for combining RNAi techniques with biological control measures to promote sustainable crop protection.[53,84] Noting that the use of microbial biocontrol agents has been discussed as a potential tool for protecting *Cannabis* crops,[20,36] the combination of RNAi technology with microbial biocontrol agents [e.g. plant growth-promoting rhizobacteria (PGPR)] should be investigated as a biosafe solution for managing pests in the cultivation of medical *Cannabis*. In other crops, inoculation with PGPR has improved yields as a result of nutrient mobilization, hormone production, disease control and enhanced stress tolerance.[36] Lyu *et al.*[36] promoted investigation into such applications for environmentally sustainable improvement of *Cannabis* yields and medicinal qualities. Studying the effects of PGPR inoculants and/or other microbial biocontrols, and their combined efficacy when used alongside RNAi technology, represents a potentially rewarding avenue of research for biosafe protection of medical *Cannabis* crops.

4 FINAL REMARKS

The expanding *Cannabis* industry would benefit from a proactive, rather than reactive (i.e. acting after the observed increase in pest pressure/expansion) approach that examines the abovementioned RNAi technologies in the context of large-scale cultivation of medical *Cannabis*. Indoor settings are simultaneously well-suited for both medical *Cannabis* cultivation (often requiring specific conditions, e.g. low humidity) and research examining technologies that are currently unapproved by state, national and multinational legislative bodies. For example, environmental release of transgenic RNAi cultivars/strains is not yet approved in European Union member states. With the recent expansions observed for indoor and outdoor *Cannabis* production, new pests of this crop are continuously being detected,[17–19] and efforts must continue to unveil and predict current and forthcoming pests that could dramatically reduce yields of the final medicinal products. Furthermore, current regional restrictions against the environmental release of certain RNAi technologies must not impede scientific progress towards RNAi-based protection of *Cannabis* crops. Different RNAi techniques should be examined against the various types of *Cannabis* pests. *Cannabis* transformation and micropropagation protocols have yet to be fully optimized for examining certain transgenic RNAi techniques, and thus these protocols require further development. Combining RNAi and biological control, especially microbial biocontrol agents, should be investigated with regard to their combined efficacy in medical *Cannabis* protection. Finally, ensuring biosafety from a human toxicological viewpoint must be a primary focus in endeavours promoting RNAi-based protection of medical *Cannabis* plants. There remains an urgent need to investigate these research avenues before the appearance of novel or previously hidden *Cannabis* crop–pest dilemmas.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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